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EDITED BY

SIMON FLEXNER, M.D. PEYTON ROUS, M.D.

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STUDIES CONCERNING THE RELATIONSHIP BETWEEN PNEUMOCOCCI AND STREPTOCOCCI.

By HOBART A. REIMANN, M.D.

(*From the Hospital of The Rockefeller Institute for Medical Research.*)

(Received for publication, July 30, 1926.)

From time to time evidence has been presented that a close relationship exists between Pneumococcus and Streptococcus. Kruse and Pansini (1), observing the variability of Pneumococcus when grown under different conditions, drew attention to the resemblance between certain races of Pneumococcus and Streptococcus and even suggested the possibility that these bacteria might have their origin in a common, probably saprophytic form of streptococci. Since then a number of observers have brought forth evidence indicating that *Streptococcus hæmolyticus*, *Streptococcus viridans*, *Streptococcus mucosus* and Pneumococcus are so closely related that under certain conditions one may change into the other. The evidence for this point of view has recently been greatly supported by the series of publications of Morgenroth and his collaborators and assistants.

Morgenroth, Schnitzer and Berger (2) have reported that by special methods it was possible to transform with great regularity pneumococci into streptococci.

The methods employed were the following: Pneumococci were cultivated in medium containing dead yeast cells or animal charcoal which had absorbed optochin. Under these conditions the pneumococci underwent modifications and were transformed into cocci which possessed properties held to be characteristic for *Streptococcus viridans*. The organisms were now insoluble in solutions of sodium taurocholate, were avirulent for mice, were resistant to the action of optochin and were more sensitive to the action of rivanol than was the original strain. According to these writers, this transformation of pneumococci into streptococci did not occur suddenly but in undergoing the transformation the bacteria passed through several stages or modifications.

Bacteria in the first stage of the process were described as Modification A. While the bacteria in this stage retained most of the characteristic properties of pneumococci, they had become more resistant to the action of optochin. When blood agar plates were made from the Modification A culture, after growing in optochin, it was found that the colonies no longer resembled those of the original culture but now resembled the colonies of *Streptococcus viridans*. Microscopically the organisms appeared as long chains of round individuals; they were bile-insoluble and were only killed by very high concentrations of optochin. This form was called Modification B and was considered to be identical with *Streptococcus viridans*. Upon further growth in artificial medium, or in the animal, and occasionally upon growth in optochin-containing medium, the third form, Modification C, was found to arise. These organisms corresponded in their properties to *Streptococcus hæmolyticus*, that is, they grew on blood agar with more or less hemolysis, were bile-insoluble and optochin-fast. When injected into the subcutaneous tissue of the abdomen of the mouse phlegmons formed. Moreover, these organisms showed the sensitiveness to the action of rivanol which is characteristic for *Streptococcus hæmolyticus*. Occasionally the transition of Modification A into Modification C occurred without passing through the intermediate stage of Modification B.

These observations concerning the changes undergone by pneumococci and streptococci are of great importance not only in their bearing on the classification of these microorganisms, but also in their relation to the pathogenesis of human infection. It has seemed of importance, therefore, to repeat those experiments and to attempt to interpret them in the light of studies made by the writer (3, 4) and others concerning the variations in the characteristics of Pneumococcus which occur under a number of conditions.

It has been found by a number of observers that when pneumococci are grown in various unusual ways, such as in immune serum or in bile, atypical colonies may appear. These have been called R, or rough, colonies in contrast with the S, or smooth, colonies which are the typical pneumococcus colonies. The bacteria of the R colonies are avirulent for mice, have no capsule, do not produce the so called pneumococcus soluble substance, are somewhat resistant to the action of bile and have lost their type specificity. They have been found to retain their properties on further cultivation and even after repeated passage through mice. Blake and Trask (5) have described a third type of colony, probably made up of intermediate forms, the bacteria of which may revert to the typical virulent pneumococci on passage

through mice. The R bacteria although to some degree resembling streptococci of the *viridans* variety have nevertheless been considered by the writer and by most of the workers on this problem to be modified pneumococci.

Methods.

In studying the modifications undergone by pneumococci when exposed to the action of optochin or optochin absorbed on yeast or on animal charcoal, the following methods were employed.

A strain of Pneumococcus derived from a single cell of a Type I stock culture was employed. 0.05 cc. of a 1–10 dilution of an 18 hour broth culture of this organism was inoculated into serum bouillon containing varying concentrations of optochin hydrochloride (ethyl hydrocupreine hydrochloride, Zimmer and Co.) or into serum broth containing varying concentrations of optochin absorbed on yeast. These cultures were incubated from 18 to 20 hours and plated on blood agar to detect colony variations. Yeast-absorbed optochin was prepared as follows: A 10 per cent suspension of dried yeast (Fleischmann) in distilled water was made and this was sterilized by heat and then centrifuged. The supernatant fluid was replaced with a 1–10,000 solution of optochin hydrochloride in distilled water and the mixture incubated ½ hour. The suspension was again centrifuged, the supernatant fluid discarded and replaced by twice the amount of 10 per cent serum broth.

For testing bile solubility, 0.5 cc. of sterile, undiluted ox bile was added to 0.5 cc. of a broth suspension of bacteria and incubated for 2 hours at 37°C.

Experimental.

An 18 hour broth culture of Pneumococcus was inoculated into broth containing a suspension of yeast-absorbed optochin as described above and incubated 20 hours. After plating on blood agar only one kind of colony appeared and this resembled exactly the S form of colony which has been described in a previous paper (3). The original culture was now passed two more times through the yeast-absorbed optochin medium. From this third culture, plates were made and now a number of small, thin, rough surfaced colonies, like those which have previously been described as R forms, appeared. A study was next made of the changes undergone by pneumococci when grown in broth containing various amounts of optochin without any yeast being present, and also when grown in suspensions of yeast containing no optochin. The culture was grown repeatedly in serum broth containing optochin hydrochloride in concentration of 1–100,000. At the ninth transfer the concentration was increased to 1–50,000 and at the thirtieth transfer, to 1–20,000. Upon plating the culture at intervals during the repeated transfers, no small R colonies appeared. This last experiment was repeated, substituting plain broth for serum broth. Three transfers were made in this medium con-

taining optochin hydrochloride in a concentration of 1–600,000. When this third culture was plated on blood agar, three kinds of colonies were found, S colonies, R colonies and colonies of an intermediate form like those described by Blake and Trask.

The culture was also grown repeatedly in a 10 per cent suspension of heat-killed yeast in serum broth, no optochin being present. Transfers were made at 6 hour intervals on account of the tendency of the organisms to die in this medium. After the fourth transfer in this medium, plating on blood agar showed a great preponderance of colonies of the R form over those of the S forms.

These experiments, which have been repeated a number of times, indicate that in our hands the result of growing typical pneumococci in media containing yeast which has absorbed optochin, or even in media containing optochin alone or yeast alone, is the appearance of modified pneumococci which form colonies identical with those described by a number of writers as R colonies. From the description of the colonies of Modification B as given by Morgenroth and his associates, it seems probable that the bacteria of Modification B are identical with those we and others have described as pneumococci of the R colonies.

Morgenroth and his collaborators consider these to be true streptococci of the *viridans* type. We have called them modified pneumococci. It is true, as Morgenroth and his collaborators state, that these colonies resemble those of *Streptococcus viridans*. Moreover, the bacteria from these colonies have little virulence for mice and they are resistant to optochin and do not dissolve as readily as do typical pneumococci in solutions of bile. The writers we have mentioned state that these organisms are insoluble in solutions of sodium taurocholate and they lay stress on this property as indicating their identity with the bacteria of the *Streptococcus viridans* group. In carrying out the bile test, however, Morgenroth and his associates have employed the method of Levy (6), adding a 2.5 per cent solution of sodium taurocholate to the broth culture. We have modified this technic, employing not only a 2.5 per cent solution of this substance, but also solutions of a higher concentration. The effect of ox bile itself on these organisms has also been tested. Many tests of R cultures have been made, and at the same time tests have been made of twelve different cultures from various sources which had previously been identified by another worker as cultures of *Streptococcus viridans*.

The latter cultures were all found to be insoluble in ox bile and also in sodium taurocholate solutions, even when this was highly concentrated. On the other hand, when the bacteria of the R colonies or what Morgenroth and his associates have called Modification B bacteria, were tested, it has been found that although they are resistant to the action of sodium taurocholate in 2.5 per cent solution, they are dissolved if higher concentrations of this salt be employed. Moreover, if the bacteria be suspended in salt solution instead of in broth, solution occurs even in 2.5 per cent solutions of sodium taurocholate. Finally, if ox bile be employed, these organisms all dissolve, although more slowly than usually occurs with typical pneumococci. Our experiments, therefore, indicate that these modified bacteria are not, properly speaking, bile-insoluble; they possess increased resistance to the action of bile. They, therefore, differ in this respect from bacteria of the *Streptococcus viridans* group which are truly bile-insoluble. They also differ from *Streptococcus viridans* in certain other properties. We have previously noted the readiness with which pneumococci undergo autolysis when they are suspended in saline solutions and kept at 37°C. This readiness to undergo autolysis is seen in pneumococci of all types and is especially well marked in bacteria from the R colonies. On the other hand, streptococci of all varieties undergo autolysis very slowly.

Saline suspensions of six strains each of the S and R forms of pneumococci and of ten strains of typical *Streptococcus viridans* were made and placed in the incubator at 37°C. for 48 hours. At the end of this time the tubes were centrifuged and stained films were prepared from the sediment of each tube. It was found that well formed, Gram-positive cocci were still present in all the tubes containing streptococci, while in the tubes containing the S and R forms of pneumococci no intact cells remained.

We have reported elsewhere (7) that freezing and thawing easily causes disruption of the cell bodies of pneumococci, while this treatment has no effect on streptococci. Pneumococci of the R form obtained by growth in serum and in bile and also modified pneumococci obtained by growth in media containing optochin absorbed on yeast have been tested by these methods. In every instance all these

bacteria have been found to be readily disintegrated by the action of freezing and thawing.

Finally, the immunological properties of atypical pneumococci derived from type-specific strains by growth in broth containing yeast-absorbed optochin were compared with the immunological properties of typical streptococci of the *viridans* group.

We have previously reported observations concerning the immunological reactions of the R forms of pneumococci (4). It was there shown that sera prepared with each of these strains agglutinated all other R forms, no matter how obtained or from what type of Pneumococcus. These sera also caused precipitation of the protein derived from all kinds of pneumococci. The interpretation placed on these experiments was that the R forms of pneumococci were pneumococci which had lost the property of producing the pneumococcus type-specific carbohydrate substance.

Immune sera were now prepared by injecting rabbits with cultures of *Streptococcus viridans*. Six different strains were employed, an immune serum being produced against each one of these strains. These special strains were selected because they grew diffusely in broth. Each of these sera was found to be specific for the homologous organism, causing agglutination usually in dilutions as high as 1–320. In the concentrated serum there occurred some crossing or non-specific agglutination of the other *Streptococcus viridans* strains. All but one of the sera also agglutinated suspensions of R pneumococci but the agglutinating power for these organisms was not as great as that of the serum prepared by the injection of R pneumococci. By absorption of the agglutinins for the R strains by R organisms the agglutinins of the antistreptococcus sera for heterologous *Streptococcus viridans* strains were removed and the absorbed sera were now race-specific and agglutinated only the homologous race of *Streptococcus viridans*. Anti-R pneumococcus sera, on the other hand, did not agglutinate any of the six strains of *Streptococcus viridans* studied.

The following interpretation of these results, in the light of the work of Heidelberger and Avery (8) seems justifiable. Lancefield (9) has previously shown that the protein of various strains of *Streptococcus viridans* is immunologically identical with that of Pneumococcus. Individual strains of *Streptococcus viridans* also possess a substance

comparable to that of the soluble specific substance of Pneumococcus and this endows them with the power of reacting specifically with their homologous serum. To what degree a single specific substance is common to a number of *Streptococcus viridans* strains, only further work will show. The protein of all strains being alike and being common to all pneumococci, the serum produced by each *Streptococcus viridans* strain agglutinates all R pneumococci. The anti-R serum, however, does not agglutinate the *Streptococcus viridans* strain because each of these strains has a specific reacting substance, which masks or prevents the non-specific antiprotein reaction. This is analogous to the immunological phenomena exhibited by the specific pneumococci. These organisms, although they contain the non-specific pneumococcus protein, are not agglutinated by anti-R serum or by anti pneumococcus-protein serum. The explanation offered for the immunological phenomena exhibited by strains of the *Streptococcus viridans* group also receives support from the work of Zinsser on hemolytic streptococci (10).

It seems from all of this work that the atypical forms of bacteria obtained by growing type-specific strains of pneumococci in media containing serum or bile or by growing them in dilutions of optochin, or in yeast-absorbed optochin according to the method of Morgenroth, are not strains of *Streptococcus viridans* but are in fact degraded forms of pneumococci.

The reasons we have adduced for this are as follows:

1. Degraded forms of pneumococci are not bile-insoluble, as Morgenroth and his associates have stated, although they are more resistant to bile than are typical pneumococci.

2. Degraded forms of pneumococci behave like typical pneumococci and not like streptococci in the readiness with which they undergo autolysis and in the ease with which the cell bodies are broken down by freezing and thawing.

3. Immune sera produced by the injection of animals with degraded strains of pneumococci behave toward typical strains of *Streptococcus viridans* just as they do toward specific strains of Pneumococcus. Typical strains of *Streptococcus viridans* possess a type-specific immunological mechanism just as do typical pneumococci. The species-specific immunological mechanism possessed by *Streptococcus viridans*

is identical with that of Pneumococcus. To this extent Pneumococcus and *Streptococcus viridans* are closely related. Whether a typical type-specific strain of Pneumococcus, however, is ever changed into one of *Streptococcus viridans* any more than a Pneumococcus of Type I is ever changed into one of Type II or of Type III only further work will show. The evidence for this at the present time is not convincing.

Experiments with Hemolytic Streptococci.

Morgenroth and his associates (11) also report that they have been able, by special methods, to convert strains of *Streptococcus hæmolyticus* into those of *Streptococcus viridans*. This change was easily induced by cultivating hemolytic streptococci in serum broth containing rivanol bound to erythrocytes, "*haut-pulver*," animal charcoal or yeast. Rivanol solutions alone in serum broth were not efficacious. Rivanol (2-ethoxy-6, 9-diaminoacridium hydrochloride) is a salt which is said to have a specific germicidal action for *Streptococcus hæmolyticus*.

Other investigators have also observed hemolytic streptococci change into non-hemolytic forms. Valentine and Krumwiede (12) on one occasion observed the spontaneous appearance of occasional green colonies on a blood agar plate made from a culture of *Streptococcus hæmolyticus* (Strain 32, Dochez, Avery and Lancefield (13)). Bacteria from both kinds of colonies were agglutinable in the original strain antiserum and each absorbed the common antibody. Lehmann (14) obtained green-producing colonies from 3 out of 87 strains of hemolytic streptococci which had been cultivated in milk media.

On account of the bearing which these observations concerning streptococci have on the preceding experiments, it seemed of interest to repeat the experiments of Morgenroth and his associates.

Methods.

A 10 per cent suspension of dry yeast in distilled water was sterilized and centrifuged as described previously. The supernatant fluid was replaced with a 1–1,000 solution of rivanol (Metz) in distilled water and incubated for 1 hour at 37°C. After centrifuging, the sediment was washed three times with distilled water and made up to the original volume. Varying dilutions of this suspension were made in 10 per cent serum broth. 0.05 cc. of a 1–10 dilution of a broth culture of *Streptococcus hæmolyticus* was inoculated into 2 cc. of the respective dilutions of yeast-absorbed rivanol and incubated at 37°C. for 20 hours.

In order to obtain pure line cultures, in certain of the experiments single chains of 3 or 4 cocci were isolated according to the technic described in a previous paper (3). Single cocci are difficult to isolate on account of their small size and resemblance to particulate matter in the agar.

Experimental.

Twenty-six strains of hemolytic streptococci, including stock cultures and freshly isolated strains, were tested. Bacteria were inoculated in serum broth containing rivanol alone and in serum broth containing yeast alone, but in most instances in dilutions of rivanol-yeast suspensions varying in strength from 1–4 to 1–8,000. The organisms tended to die in the stronger concentrations and for prolonged growth required alternate transfers into plain serum broth. Cultures could be transferred indefinitely in dilutions weaker than 1–1,000.

After each transfer the organisms were plated on blood agar, incubated at 37°C. and the plates examined for the presence of atypical colonies. Approximately, 400 transfers were made in the various media and only on the plates made from one of the strains (Streptococcus 267) were atypical colonies seen. The variant colonies which occasionally appeared on the blood agar plates among the hemolytic colonies of this strain were anhemolytic, and the organisms derived therefrom were avirulent and bred true on further culture. However, atypical colonies appeared a number of times spontaneously when this culture, without any previous treatment with yeast-absorbed rivanol, was plated on blood agar.

To eliminate the possibility of the variant being a contaminant, 20 single short chains were isolated from the original culture and treated with yeast-absorbed rivanol in dilutions varying from 1–1,000 to 1–8,000. Under these conditions no variant colonies appeared. This experiment does not entirely prove that the variants were contaminants but this explanation seems not improbable.

Two of the original cultures, S3 and S23, were transferred 70 times on plain agar before treatment with yeast-absorbed rivanol. In spite of this preliminary treatment no variants ever appeared.

SUMMARY AND CONCLUSIONS.

Morgenroth and his collaborators grew pneumococci in a medium containing optochin and derived variant forms of bacteria therefrom which were considered to be streptococci of the *viridans* group. We have repeated these experiments and have also derived variant forms. These atypical races, however, we have found to be identical with the R form of pneumococci which have been obtained by various other methods. That these R cultures are still pneumococci and do not belong in the *Streptococcus viridans* group is supported by the following observations:

1. These strains are not bile-insoluble, but are more resistant to the lytic action of this agent than are type-specific pneumococci.

2. R strains behave like pneumococci and not like streptococci in the readiness with which cultures spontaneously autolyze and saline suspensions disintegrate during freezing and thawing.

3. Immunological reactions of the variant pneumococci derived by Morgenroth's method are identical with the immunological reactions of R forms of pneumococci derived by various other means.

The observations of Morgenroth and his associates in regard to the transformation of *Streptococcus hæmolyticus* into *Streptococcus viridans* by treatment with rivanol could not be repeated in this laboratory. No explanation is at hand for our failure to produce the change. It is of course possible that none of the twenty-six strains tested had suitable tendencies to variation or that the technic or reagents employed varied from those of Morgenroth.

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EFFECT OF REPEATED FREEZING (−185°C.) AND THAWING ON COLON BACILLI, VIRUS III, VACCINE VIRUS, HERPES VIRUS, BACTERIOPHAGE, COMPLEMENT, AND TRYPSIN.

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The effect of low temperatures on bacteria, viruses, ferments, cells, and the bacteriophage has been observed and reported. The effect of repeated freezing and thawing on some of them has also been studied. The temperatures used, however, as well as the number of freezings and thawings employed, varied greatly, and in many instances the effect on only one or two agents was studied by the same worker. Nevertheless some observers have stated that by means of repeated freezing and thawing it is possible to determine whether certain of these agents are living or dead. In view of the inadequacy of the knowledge concerning this subject it seemed desirable to study further the effect of repeated freezing (−185°C.) and thawing on vaccine virus, Virus III, herpes virus, and the bacteriophage as compared with the effect on colon bacilli, complement, and trypsin. The results of the investigation are reported in this paper.

Macfadyen (1–3) exposed vegetative and spore forms of bacteria, seed, and Buchner's zymase to the temperatures of liquid air (−185°C.) and liquid hydrogen (−252°C.) for 20 hours and found that the bacteria grew, that the seed germinated, and that the zymase was still active. No determinations of the number or amount of the active agents, however, were made before or after the exposures.

White's (4) report concerning the results obtained by exposing bacteria to the temperature of liquid air for a period of 2 hours shows that 50 per cent of the hardier bacteria survived while 98 per cent of the less resistant varieties were killed. He also states that vaccine virus was active after an exposure for 15 minutes to the temperature of liquid air. No determinations of the amount of active virus were made, however, before or after the exposure.

Barratt (5) found that rabies virus was active after an exposure for 3 months

to the temperature of liquid air. The same virus, however, was not active after treatment for 45 minutes in a disintegrating machine kept at a temperature of −185°C.

Salvin-Moore and Barratt (6) exposed cells of a graftable mouse cancer to the temperature of liquid air for 30 minutes and found that mice inoculated with the treated cells developed cancers. The observers assume that the cells were killed by the treatment while the cancer virus survived.

Gaylord (7) found that cells of a transplantable mouse cancer, although injured, were able, after exposure for 80 minutes to the temperature of liquid air, to produce tumors when injected into mice. On the other hand, he found that freezing, by means of liquid air, killed embryonic tissue. He also observed that trypanosomes were able to resist a temperature of −185°C. for a period of 20 minutes but not for a period of 40 minutes.

Stockman and Minett (8) state that the virus of foot and mouth disease is active after repeated freezing and thawing. Ammonia brine was used for freezing.

Sanderson (9), using two strains of bacteriophage, one active for staphylococci, the other lytic for colon bacilli, found that the titer of the phages was not influenced by freezing and thawing 20 successive times. Solid CO_2, approximately −78°C., was used for freezing. The results of his experiments lead him to believe that the bacteriophage is something other than a viable organism.

D'Hérelle (10), working with two strains of bacteriophage, one active for dysentery bacilli, the other lytic for staphylococci, found that the phage in filtrates less than 17 days old resisted at least 3 exposures to the temperature of liquid air for periods of 10 minutes each, while the phage in older filtrates was not active after 1 to 3 such exposures.

Methods and Materials.

Freezing and Thawing.—The low temperature (−185°C.) used in the experiments to be reported was obtained by means of commercial liquid air which was transported from the plant to the laboratory in Dewar flasks. Desired amounts of the air were transferred to deep Dewar beakers where small amounts of the substances to be frozen, enclosed in Noguchi tubes, were completely immersed for several minutes. After the substances had been completely frozen they were quickly thawed in tap water (16–18°C.). This procedure was repeated as often as desired. Then the treated substances, as well as the controls, were tested and titrated by standard methods.

B. coli.—Colon bacilli, 24 hours old, were washed off agar slants and suspended as desired in water, broth, physiological salt solution, or Locke's solution.

Vaccine Virus.—Testicles, into which vaccine virus had been injected 4 days previously, were removed from the rabbit and ground up with sand. After the addition of 20 cc. of Locke's solution, the emulsion was centrifuged, and the supernatant fluid was used as a stock virus. A fresh stock was made for each experiment.

Virus III.—Virus III (11), indigenous to rabbits, was prepared for each experiment in a manner similar to that described for vaccine virus.

Herpes Virus.—Herpes virus was injected into the brain* of a young rabbit. The animal usually died 5 days later. From the brain, removed immediately after death, a 1–10 emulsion was made in Locke's solution and centrifuged. The supernatant fluid was used as a stock virus. Fresh stock was made for each experiment.

Bacteriophage.—A bacteriophage lytic for *B. coli* was prepared in the usual manner in buffered broth with colon bacilli. The resistent bacteria were removed by filtration and the filtrate was used as a stock phage. The filtrates used in the experiments were 19 to 90 days old and the lytic principle in them was usually active in a dilution 10^{-10}. Titrations for the activity of the phage were made in the usual manner by means of dilution with broth in which young colon bacilli were suspended. After the different dilutions were incubated for 48 hours, the cloudy tubes were heated at 60°C. for 30 minutes, and then 0.5 cc. from each tube was tested for the presence of phage. In this way the last tube in which phage was present could be accurately determined.

Complement.—Complement in fresh guinea serum was used. Dilutions of the serum were made by means of physiological salt solution. Activity of the complement was tested in the usual way by dilution or by the determination of the length of time required by the complement to hemolyse a known amount of red blood cells. Care was taken not to inactivate any of the complement by shaking.

Trypsin.—Trypsin, in a buffered solution of pH 7, was supplied by Dr. Northrop. Determinations of the ferment's activity before and after freezing and thawing were made by Dr. Northrop. Details of the technic can be obtained from his papers (12). Care was taken to inactivate none of the ferment by shaking.

EXPERIMENTAL.

All experiments made to determine the effect of freezing (−185°C.) and thawing on colon bacilli, Virus III, vaccine virus, bacteriophage, complement, and trypsin were repeated several times, and many of them were performed simultaneously. For convenience, however, the results of the experiments will be reported separately.

Colon Bacilli.

It is a well established fact that many bacteria are killed by repeated freezing and thawing, even when a temperature only a few degrees below 0°C. is employed. Therefore colon bacilli were in-

* All operations were performed under ether anesthesia.

cluded as a control in the list of active agents to be exposed repeatedly to the temperature of liquid air, and the following experiment is illustrative of the results obtained with these organisms.

Colon bacilli, washed from an agar slant and suspended in broth, were frozen and thawed 12 successive times. Before freezing there were 180,000,000 viable organisms per cc. After freezing and thawing, however, only 40,000 organisms per cc. remained viable.

When it had been shown by the experiment above that colon bacilli are killed by repeated exposure to the temperature of liquid air, the following experiment was performed to determine what influence the number of bacteria per cc. and the type of suspending fluid have on the percentage of organisms killed by repeated freezing and thawing.

TABLE I.

Summary of Experiment Showing the Effect of Dilution and Diluent on the Percentage of Colon Bacilli Killed by Freezing and Thawing 4 Successive Times.

Diluent	Dilution	No. bacteria per cc. before freezing	No. bacteria per cc. after 4 freezings and thawings
Locke's solution	1–10	18,400,000	27,000
" "	1–1,000	380,000	20
Broth	1–10	44,000,000	13,200,000
"	1–1,000	600,000	188,000

Colon bacilli, removed from an agar slant, were suspended in sterile distilled water. 0.5 cc. of the suspension was put in 4.5 cc. of buffered broth and Locke's solution respectively. Further dilutions, 1–1,000, were made with each diluent, and two of the suspension, 1–10 and 1–1,000, in each series were frozen and thawed 4 times. The number of viable organisms per cc. was determined before and after freezing. The results of the experiment are summarized in Table I.

It is obvious from the results shown in Table I that the colon bacilli were more susceptible to repeated freezing and thawing when Locke's solution instead of broth was employed as a suspending fluid. It is also evident that approximately the same percentage of bacteria was killed in the different suspensions made by means of broth. On the other hand, a greater percentage of bacteria was killed in the 1–1,000 dilution made with Locke's solution than in the 1–10 dilution made with the same fluid.

Virus III.

The effect of repeated freezing and thawing on Virus III was determined by means of the following experiments.

1 cc. of a fresh stock emulsion containing active Virus III was added to 9 cc. of Locke's solution. One half of the emulsion was used as a control; the other was frozen and thawed 22 successive times. From the second half samples were removed for tests after the 2nd, 12th, and 22nd freezings. The control emulsion and the samples of the treated one were tested for the presence of active Virus III by means of inoculations in the shaved skin of the same normal rabbit. The results of the tests are shown in Table II.

TABLE II.

Summary of the Results Obtained by Repeated Freezing and Thawing of Virus III.

No. of freezings and thawings	Dilutions		
	1–10	1–100	1–1,000
Control (unfrozen)	++	+±	+
2	++	+	±
12	−	−	−
22	−	−	−

+'s indicate the presence of a virus reaction at the sites of inoculation. − indicates the absence of a reaction. Dilutions are in terms of the stock testicular emulsion containing the virus.

The experiment indicates clearly that Virus III is easily killed or inactivated by repeated freezing and thawing in spite of the high protein content of the emulsion containing the virus.

Vaccine Virus.

Vaccine virus in a stock testicular emulsion, diluted 1–10, 1–100, and 1–1,000, showed no appreciable decrease in its titer after 12 successive freezings and thawings. Finally, however, it was possible as will be shown in the following experiment, to decrease the titer of the virus by repeated freezing and thawing.

1 cc. of a stock testicular emulsion containing active vaccine virus was diluted 1–10, 1–100, and 1–1,000 by means of Locke's solution. One half of the 1–1,000 dilution was used as a control; the other half was frozen and thawed 34 successive

times. Samples of the latter half were removed for tests after the 24th and 34th freezings. The control emulsion and the samples of the treated one were tested for the presence of active vaccine virus by means of inoculations in the shaved skin of the same normal rabbit. The results of the tests are summarized in Table III.

From the results of the experiment just described it is evident that a certain percentage of the vaccine virus, diluted 1–1,000 with Locke's solution, was inactive after 34 successive freezings and thawings. This virus, however, proved to be more resistant than any other of the active agents tested.

TABLE III.

Summary of the Results Obtained by Repeated Freezing and Thawing of Vaccine Virus.

No. of freezings and thawings	Dilutions		
	1–1,000	1–10,000	1–100,000
Control (unfrozen)	+++	++	+
24	++	+	±
34	++	+	−

+'s indicate the presence of a virus reaction at the sites of inoculation. − indicates the absence of a reaction. Dilutions are in terms of the stock testicular emulsion containing the virus.

Herpes Virus.

The activity of herpes virus in a fresh stock emulsion was not appreciably decreased by 12 successive freezings and thawings. The following experiment, however, shows that the virus is susceptible to such treatment under certain conditions.

0.5 cc. of a fresh stock brain emulsion containing active herpes virus was diluted 1–20 by means of Locke's solution. One portion of the diluted emulsion was used as a control; another portion was frozen and thawed 24 times. The two portions were then tested for the presence of active virus by means of intracerebral inoculations in young rabbits. The results of the tests are summarized in Table IV.

The experiment demonstrates that herpes virus, provided the brain emulsion containing it has been sufficiently diluted by means of Locke's solution, is killed or inactivated by repeated freezing and thawing.

Bacteriophage.

Early in the work with a bacteriophage lytic for colon bacilli it became evident that repeated freezing and thawing inactivated at least 99 per cent of the phage. Details of these experiments will be omitted since results similar to the ones obtained in them will be

TABLE IV.

Summary of Results Obtained by Repeated Freezing and Thawing of Herpes Virus.

No. of freezings and thawings	Dilutions		
	1–20	1–40	1–80
Control (unfrozen)	+	+	+
24	−	−	−

+ indicates death of the animal within 7 days after intracerebral inoculation of 0.25 cc. of the emulsion diluted as noted. − indicates that the animal was alive 4 weeks after inoculation. Dilutions are in terms of the stock emulsion.

TABLE V.

Summary of Experiment Showing the Effect That the Type of Diluent Has on the Percentage of Bacteriophage Inactivated by Freezing and Thawing.

Diluent	Dilutions of stock filtrate frozen and thawed	Titer of phage after 12 freezings and thawings
Broth (unfrozen control)	1–10,000	10^{-11}
"	1–10,000	10^{-7}
Distilled water	1–10,000	10^{-6}
Locke's solution	1–10,000	10^{-5}
Salt "	1–10,000	Completely inactivated

Dilutions are in terms of the stock filtrate.

shown in other experiments performed to ascertain what influence the degree of dilution or the type of diluent has on the percentage of phage inactivated by repeated freezing and thawing. The effect that the type of diluent has on the percentage of phage inactivated under these conditions was investigated first in the following manner.

Portions of the stock filtrate containing the active lytic substance were diluted 1–10,000 respectively by means of buffered broth, Locke's solution, distilled

water, and physiological salt solution. The pH of the first two diluents was 7.6, while that of the last two was between 7 and 6. Small amounts of the diluted filtrate were frozen and thawed 12 successive times. Then tests were made in the usual way to determine the titer of active phage in the control as well as in each treated specimen. The results of the titrations are shown in Table V.

The experiment affords conclusive evidence (1) that phage is inactivated by repeated freezing and thawing, and (2) that the type

TABLE VI.

Summary of Experiment Showing the Effect That Dilution Has on the Percentage of Bacteriophage Inactivated by Freezing and Thawing.

Diluent	Dilutions of stock filtrate frozen and thawed	Titer of phage before and after 8 freezings and thawings
Broth (control)	1–10	10^{-11}
" (frozen)	1–10	10^{-9}
" "	1–1,000	10^{-9}
" (control)	1–1,000	10^{-11}
Distilled water (control)	1–10	10^{-11}
" " (frozen)	1–10	10^{-8}
" " "	1–1,000	10^{-8}
" " (control)	1–1,000	10^{-10}
Locke's solution (control)	1–10	10^{-11}
" " (frozen)	1–10	10^{-10}
" " "	1–1,000	10^{-7}
" " (control)	1–1,000	10^{-11}
Salt solution (control)	1–10	10^{-11}
" " (frozen	1–10	10^{-9}
" " "	1–1,000	10^{-5}
" " (control)	1–1,000	10^{-11}

Dilutions are in terms of the stock filtrate.

of diluent influences the percentage of phage inactivated in this manner.

After it had been determined that the type of diluent does affect the percentage of phage inactivated by repeated freezing and thawing, the following experiment was performed to ascertain what effect the degree of dilution has on the percentage of phage inactivated by such treatment.

Portions of the stock filtrate containing phage were diluted 1–10 and 1–1,000 by means of each of the following diluents: broth, distilled water, Locke's solution, and physiological salt solution. Half of each specimen was used as a control; the other half was frozen and thawed 8 times. Tests were then made in the usual way to determine the titer of active phage in each specimen. In these titrations pipettes were changed after every third tube. Consequently the titer of the phage in each series may be slightly higher than it would have been had a clean pipette been used after each tube. Since the same technic was used throughout the experiment, the results of the different titrations summarized in Table VI are comparable.

The results of the experiment indicate that an increase in the dilution of the stock filtrate by means of broth and distilled water

TABLE VII.

Summary of Experiment Showing the Effect That Freezing and Thawing Have on Complement in Diluted Serum.

Specimens of complement	Titration by dilution				Time required for complete hemolysis*
	1–50	1–100	1–200	1–400	
1–50 (control)	++	+	±	−	3 min.
1–50 (frozen and thawed)	+	±	−	−	7 "
1–100 (control)		+	±	−	4 "
1–100 (frozen and thawed)		−	−	−	No hemolysis

* Dr. Landsteiner was kind enough to make this test.

+'s indicate hemolysis. − indicates no hemolysis.

does not at the same time lead to an increase in the percentage of phage inactivated by repeated freezing and thawing. On the other hand, an increase in the dilution accomplished by means of physiological salt solution and Locke's solution does lead to an increase in the percentage of phage inactivated, as evidenced by a greater percentage of phage being inactivated in dilutions of 1–1,000 than in dilutions of 1–10. These findings are similar to the ones shown in Table I concerning the effect of dilution on the percentage of bacteria killed by repeated freezing and thawing.

Complement.

The titer of complement in undiluted serum and in serum diluted 1–10 is not appreciably decreased by 12 successive freezings and

thawings. When this fact became evident, a study concerning the effect of freezing and thawing on complement in highly diluted serum was made in the following manner.

Portions of fresh guinea pig serum were diluted 1–50 and 1–100 by means of physiological salt solution. Half of each specimen was used as a control; the other half was frozen and thawed 12 times. Then tests for the presence of active complement in each of the control and treated specimens were made by means of dilution and also by the determination of the time necessary for given amounts of the sera to completely hemolyse given amounts of red blood cells in the presence of a great excess of amboceptor. Throughout the experiment, the results of which are shown in Table VII, care was taken not to inactivate the complement by shaking.

From the results of the experiment it may be concluded that complement in highly diluted serum is inactivated by repeated freezing and thawing.

Trypsin.

The effect of freezing and thawing on trypsin was studied in the following manner.

Portions of a 5 per cent stock solution of trypsin were diluted 1–10 and 1–500 by means of a buffered solution having a pH of 7. Half of each specimen was used as a control, the other half was frozen and thawed 12 times. Care was taken to inactivate none of the ferment by shaking. After the amounts of active trypsin in the control and treated specimens were determined[1] and compared, it was found that 70 per cent of the trypsin was inactivated in the specimen diluted 1–500, while only 17 per cent was inactivated in the one diluted 1–10.

The results of the experiment above afford conclusive evidence that trypsin is inactivated by repeated freezing and thawing and under these conditions a greater percentage is inactivated in a dilute than in a concentrated solution.

DISCUSSION.

The numerous discussions concerning the nature of the bacteriophage have led many investigators to question more closely the living nature of some of the so called filterable viruses. Many tests have

[1] Dr. Northrop supplied the trypsin and determined the amount of active trypsin in the control and treated specimens.

been devised to act as criteria for the presence of life, but so far no one of them has been found satisfactory. Recently Sanderson (9), using a temperature of −78°C., found no diminution in the titer of two strains of phage subjected to 20 successive freezings and thawings. Since bacteria and cells are killed by repeated freezing and thawing he concludes that the bacteriophage must be something other than a living organism. In the experiments reported in the present paper a number of active agents, some undoubtedly living, others equally unquestionably not living, and still others of a doubtful nature, were subjected to repeated freezing (−185°C.) and thawing. By these tests it has been possible to determine that mere destruction or inactivation of a substance cannot be accepted as proof that it existed in a living state.

CONCLUSIONS.

Colon bacilli, Virus III, vaccine virus, herpes virus, bacteriophage, complement, and trypsin are either killed or inactivated by repeated freezing (−185°C.) and thawing. As might be expected, some of the agents are more resistant than others.

Hence it may be concluded that destruction or inactivation of an active agent by repeated freezing (−185°C.) and thawing is not proof that it was possessed of life.

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THE RELATIVE REACTION WITHIN LIVING MAMMALIAN TISSUES.

VIII. On the Course of the Tissue Acidosis Secondary to Blood Acidosis Induced with Hydrochloric Acid.

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In a previous paper (1) the fact has been demonstrated that the reaction of certain tissues can be altered by injecting into the blood amounts of acid or alkali that are compatible with life. Rats vitally stained with a phthalein and receiving dilute hydrochloric acid survive despite marked changes in the color of the connective tissue, tendons, and cartilage. The abnormality in pH thus attested persists for a surprisingly long period. It has seemed possible, by repeating the experiments in larger animals, to learn something of the general relation existing between the reaction of the blood and tissues. Such experiments, upon rabbits, form the main subject of the present paper. But before they are taken up it will be well to give in detail a few observations on the effect upon rats of maximum quantities of hydrochloric acid.

Preliminary Experiments on Rats.

Experiment I.—At 9.20 a.m. a male rat of 123 gm., shaved over the body 24 hours previously, was given an intraperitoneal injection of 1.85 cc. of 4 per cent phenol red isotonic with the blood, but at pH 5.6. The animal was then etherized, a cannula connected with a jugular vein, the body immersed in a warm bath of oil, and, at 10.10 a.m., N/6.35 HCl was run into the circulation. The apparatus and general technic were those employed in a previous paper (2). The hue of the rat to begin with was deeper than *jasper red* (Ridgway) and no change was observed until after 3 cc. of the acid had been given, when the shaved surface appeared somewhat yellower. When 5 cc. was in, at 10.37 a.m., it had become *coral red*. The breathing was exaggerated. By 11.05 a.m. 9 cc. had been injected and the animal was slightly lighter than *rufous*. Urine had been repeatedly voided in

large amount, at first red, then orange-red. At 11.10, with 9.5 cc. in, a movement of the animal during the course of the stormy respirations loosened the attachment of the cannula, and the vein had to be tied. The ether was discontinued and the rat taken from the bath. Within 10 minutes it was walking about. The surface tissues, now decolorizing, were still acidotic as shown by a *carrot red* color.

At 1.40 p.m. when the animal had completely decolorized a second injection of 1 cc. of phenol red was given. By 2.30 it was deeply colored, *carnelian red*. At 5.10 the injection was repeated. And now the hue was indicative of a return toward the normal, being between *jasper red* and *coral red*. During the afternoon the rat voided great quantities of intensely orange urine. Next morning, as well as 5 days later, it was in excellent condition. Healing of the neck incision was rapid.

Experiment II.—The rat weighed 124 gm. and received 1.85 cc. of the same phthalein solution used previously, soon after which it was etherized and placed in the oil bath. In the course of 1 hour and 40 minutes 14.8 cc. of N/6.35 HCl was run into a jugular vein. Toward the latter part of this period there was marked air hunger. 20 minutes after the injection,—and the ether,—had been terminated, with the animal already up and about, it was given 1.0 cc. more of the phthalein into the peritoneal cavity. Within a minute it died, as if from an embolus,—though none could be found in the pulmonary artery. A clot was present in the jugular below the tie. The color changes during the injection of acid had followed the general course of those noted in Experiment I.

Experiment III.—The test was repeated but in this instance the rat of 128 gm. was anesthetized with 1.1 cc. of 20 per cent urethane injected into the subcutaneous tissue of the back of the neck. 15 minutes later it received into the peritoneal cavity 1.9 cc. of 4 per cent phenol red at pH 7.4. When the injection of acid was begun 47 minutes had elapsed in all. During the course of the next 2 hours and 24 minutes 13.5 cc. was run in. The final hue was markedly acidotic, between *light ochraceous-salmon* and *light ochraceous-buff*. The animal died about an hour later. There was no respiratory distress at any time, in noteworthy contrast to the air hunger seen when such an experiment is done under ether.

Other similar observations were made which confirmed the findings described. When N/6.35 HCl is injected (3) the connective tissue and tendons change from yellow-pink and pinky yellow respectively to a clear orange-yellow. When the rats are stained with brom cresol purple instead of phenol red a change can be seen in the color of the cartilage, evidencing that it also has been rendered acidotic.

The Vital Staining of Rabbits with Phenol Red.

White rabbits can be brilliantly stained with phenol red by the injection into an ear vein of 5 cc. of a 4 per cent solution for every kilo of body weight. Within half an hour the body surface is an intense red, but soon afterwards decolorization begins, so rapidly is the dye eliminated. The animal behaves normally from first to last, and can be stained again and again without obvious injury. Most of the phthalein—as much as 96 per cent—appears in the urine within 24 hours of the injection.

The chest and abdomen of the rabbits were shaved a day or so beforehand in order to lessen the possibilities of error due to inflammation from abrasions. They were placed upon an electrically heated pad and were often covered lightly with a cloth to ensure further against an abnormal loss of body heat. The same apparatus was used to warm the injection fluid as in the case of rats but the tube leading to the vein passed directly from the water jacket to the cannula only a few centimeters away.

The color of the shaved body of the vitally stained rabbit tends to be slightly more alkaline than that of rats similarly treated. It varies with the individual from a very exceptional *jasper red* (Ridgway)[1] to *eugenia red* more commonly. The color changes can be appraised for purposes of record in terms of pH by a method already briefly described (4). An Autenrieth wedge filled with water is placed over the shaved and oiled surface of the abdomen or chest, care being taken not to exert pressure, and the color as thus viewed is compared with that noted when one or another of a series of similar wedges, filled with buffer solutions of known pH, colored with phenol red, is placed over the shaved surface of a control rabbit. By viewing through a slit the color of the various portions of the wedge placed on the control it is possible to obtain a match in color intensity as well as color character. Differences of much less than 0.1 pH can easily be read in this way. The wedges should be warmed to 35–38°C. The hue of the vitally stained but otherwise normal animal, as expressed on the pH scale, varies between pH 7.4 (*jasper red*) and pH 7.6+ (a hue slightly purpler than *eugenia red*). It will be seen that these individual variations have about the same magnitude as those which are known to occur in the normal blood. But needless to say our

[1] Ridgway's "Color standards and nomenclature" (Published by the Author, Washington, D. C., 1912) has been utilized in recording the colors, as in a previous paper of this series (Rous, P., *J. Exp. Med.*, 1926, xliv, 815). Wherever the hues provided by this book are mentioned in describing the findings italics will be employed.

use of pH figures to record the surface hue must not be taken to imply that the visible tissues have actually this pH, since there are many factors of complication. For one thing the surface is viewed by reflected light whereas readings of actual pH should be accomplished by transmitted light. The color of a fold of loose skin as seen by the latter method when it is pulled away from the body wall by gentle traction is of a different red from that observed on inspection of the same skin *in situ* by reflected light. It might be thought that the deeply stained blood would supply an important part of the surface color; but this is not the case. When all blood is washed out of the animal the surface is still an intense red (5); and whole thickness skin grafts stain heavily with the phthalein even during the period when they are completely avascular (6). One can safely conclude that the hue of the intact body surface in the living animal is referable to a distribution of the dye to the tissues; and it is upon this well justified assumption that our experiments are based. The precise whereabouts of the extravascular phthalein that renders the animal ruddy is another matter. So little of it is in the epidermis that this may be neglected; but much is in the tissue fluid,—as can be sufficiently shown by the change in color witnessed when the latter is forced out, as by compressing a skin fold between glass slides. A great deal is fixed upon the interstitial elements of the connective tissue (7). Whether the cells of this tissue contain any is doubtful.

To follow the changes in hue of the connective tissue, as distinct from those in the composite color of the body surface we have run a fine thread through the oiled skin here and there in regions where it was loose, as just above the groins; and at various periods of the experiment by pulling upon the thread have raised a fold and compressed it briefly between slides. The color of the cartilage could be followed in the ears when the circulation in them was good; but not infrequently it was so poor that the initial staining greatly lagged behind that of the body generally, as, too, did the changes consequent upon the administration of acid. In the rat the normal cartilage stains orange-yellow with phenol red, whereas in the rabbit it becomes pale pink. The more considerable alkalinity thus evidenced in the case of the latter animal is also encountered in the connective tissue which, in a compressed skin fold, appears a frank pink with only the faintest suggestion of yellow, not yellow-pink as in the rat.

By the technic described we were able to follow closely the extravascular changes in surface hue indicative of *relative* changes in pH; and, more roughly, the changes in color undergone by connective tissue and cartilage *in situ*. It remained to determine the reaction of the blood. This was accomplished by Hawkins' method (8).

The blood specimens were taken from the marginal ear vein directly into pipettes graduated to 1/100 cc., which had been ground to fit Luer needles. The latter were short, of 23 gauge, and they had been bent at a reentrant angle for con-

venience in piercing the vein in the direction of the ear tip. At the moment when this was to be done the operator made pressure upon the vein distal to the point of entry of the needle, releasing it at once thereafter so that the flow might be unimpeded. Since the vessels were distended by stroking or heat the specimens (0.25 cc.) must be thought of as consisting for the most part of arterial blood. They were procured in duplicate under oil and diluted in the usual way with salt solution. When the animal was heavily stained only enough phenol red had to be added to this solution to permit of the preliminary adjustment to pH 7.4. More of the phthalein proved necessary, of course, when the rabbit happened to be decolorizing. The protective layer of paraffin oil was replaced with melted paraffin (of low melting point) as soon as the blood and salt solution had been mixed. Readings were made in a colorimeter block at 38°C. The correspondence between the two tubes of any given specimen was, in our experience, absolute; and the whole procedure proved easy to carry out. The pipettes and needles required most careful cleansing else clotting was encountered. The same vein could be pierced again and again. This was essential to avoid using the ear into which phthalein had been injected.

The buffer solutions used in the wedges and to determine the reaction of the blood differed slightly in pH, as determined with the potentiometer, from the calculated values. In charting the results corrections have been made for the differences, whereas in the protocols the figures as originally read off are recorded.

The Outlying Acidosis Attending Ether or Urethane Anesthesia.

In initial tests the rabbits were anesthetized with ether or urethane and they lay on the back throughout the injection period. Even under the best of conditions and in the absence of anesthesia the mere weight of the body proved sufficient to give rise to an acidosis of the surface tissues pressed upon, as was shown by their change in color from red to orange; and when the limbs were stretched out under tension the circulation on the surface of the abdomen was frequently interfered with and some acidosis developed there as well. But even in the absence of these sources of disturbance there was frequently a local acidosis to be observed in the anesthetized rabbits. On injection with phenol red after they had been unconscious for some time they colored up much less promptly than did normal animals; and large irregular regions over the body and in the ears remained untinted after the red staining had become pronounced everywhere else. When phthalein did at length penetrate into these regions their color had an orange cast. Evidently a local acidosis had developed in them, secondarily to an interference with the blood supply. As

is well known a blood acidosis develops during ether anesthesia. But independently of any such change, there was an extravascular acidosis, an "outlying acidosis" resembling that we have described in other connections (9).

Experiment IV.—Effects of urethane. 142 cc. of N/6.35 HCl was run into the jugular vein of a male white rabbit (No. 1) weighing 1625 gm., which had been given 3 cc. of 50 per cent urethane into the peritoneal cavity 2 hours beforehand. The injection took 1 hour and 40 minutes. When 50 cc. had been given 10 cc. of 4 per cent phenol red isotonic with 0.9 NaCl and at pH 5.6 was slowly introduced into an ear vein, without intermitting the flow of acid to the jugular. The animal stained only very gradually and irregularly *jasper red*, with patches coloring up later of *coral red* here and there. As time passed and the HCl injection proceeded the color altered more and more toward orange, and had become *rufous* in some places, *apricot orange* in others, by the time all the acid was in. The determinations on the blood showed a fall in pH from 7.4 prior to the giving of the acid to 7.0 immediately afterwards. Later the surface color became still yellower, between *zinc orange* and *ochraceous-salmon*, instead of altering toward red, and the animal died 1 hour and 40 minutes after the injection had been finished. From first to last there was no air hunger despite the acidosis. The surface hue at the end corresponded with pH 6.8.

In this instance not only was the staining irregular, with relatively acidotic areas here and there, but the complete absence of a compensatory increase in the breathing attested to a depressant effect of the urethane. To the absence of this respiratory compensation death was attributable, the animal becoming more and more acidotic after the injection was stopped, instead of tending to recover as happens after ether or in the absence of any general anesthesia. Cushny and Lieb have shown that the reaction of the respiratory center is altered in rabbits deeply under urethane (10).

Experiment V.—Effects of ether. The rabbit (No. 3) weighed 1600 gm. The *first blood specimens*, taken just prior to a light etherization, were at pH 7.4+. Now the jugular vein was cannulated, and 35 minutes after the blood had been taken the acid injection was begun. When 71 cc. of N/6.35 HCl had been run in the cannula was flushed with salt solution by means of a three-way stop-cock connecting with it, and 10 cc. of 4 per cent phenol red isotonic with 0.9 NaCl and at pH 7.4 was gradually injected. The animal colored only very gradually, *jasper red* here, *coral red* there, with a streak of orange-yellow along the midline from ensiform to symphysis. As more and more acid was injected the respirations became stormy and the general color changed to *apricot orange* variegated with

rufous. After 160 cc., given in the course of 2 hours, the jugular was tied and the ether, which for some time had been almost left off, was wholly discontinued. The *second blood specimens* were now taken, reading midway between pH 6.8 and 6.9. The *surface hue* on the other hand corresponded with 7.0− to 7.0+. The animal soon got to its feet and shortly thereafter the staining lost its patchy character. During the rest of the day it appeared in good condition save for the exaggerated respirations. Several blood specimens were taken. These showed only a slight return toward a normal reaction whereas there was a more marked extravascular return as shown by the surface color. The changes find record in the chart (Chart 1).

The animal was given food and left for the night. Next morning it was reported to be decolorized and in good condition, having eaten well. Unfortunately it was placed in a cage with other animals to be brought to the laboratory, and, as it moved about amongst them, it suddenly collapsed and died, making violent respiratory efforts. Apparently its slight exertions had been sufficient to overturn the *status quo*. At autopsy no anatomical cause for death was found.

In this experiment (Chart 1), which was duplicated by another, the injection of an enormous amount of the acid solution was withstood by the animal. There was the stormy respiration usual in acidosis, a feature which had been lacking with urethane, and after the injection the animal tended to recover as had not been the case when the latter was used. The staining with phenol red, done at a time when considerable acid had been run in, disclosed a poor circulatory condition of the tissues, as shown by a tardy distribution of the dye and the presence of relatively acidotic areas over chest and abdomen. The rapid change to an even staining after the animal had come out of the anesthetic was a significant feature as was also the return of the surface color toward the normal during a period when the blood reaction remained almost unchanged. As the later work showed, this return was referable to the disappearance, as the circulation became better, of a generalized "outlying acidosis" which had been present in addition to the extravascular acidosis induced by the HCl. In none of the later experiments, in which a local anesthesia took the place of a general one, was any pronounced color patching noted like that with ether or urethane; in none did the hue become as orange,—despite the fact that the injection of acid was pushed to the extreme; and in none did the extravascular reaction return more rapidly to the normal than that of the blood.

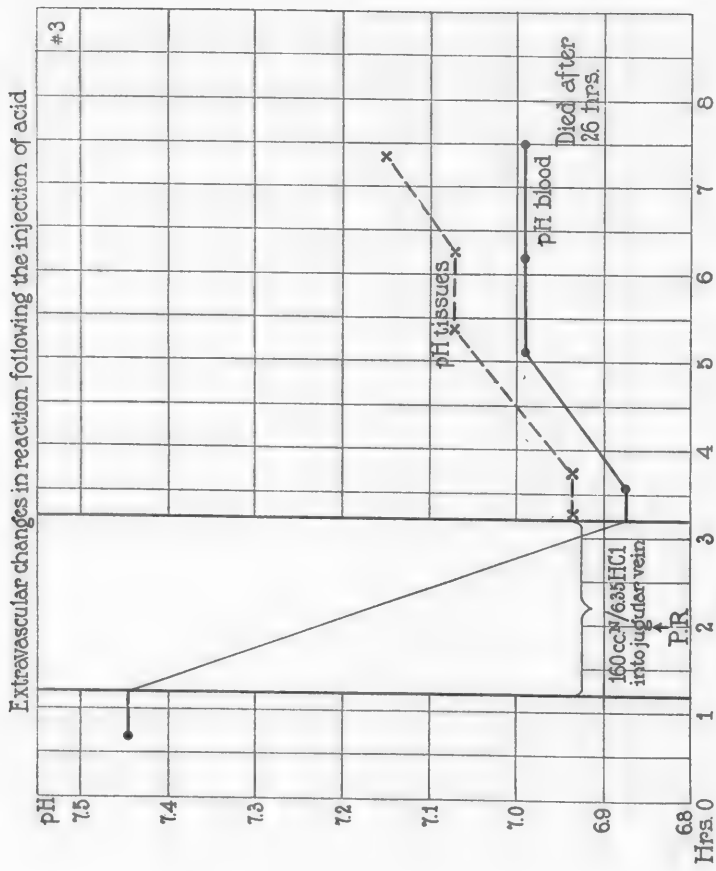
Extravascular changes in reaction following the injection of acid
#3
pH
7.5
7.4
7.3
7.2
7.1
7.0
6.9
6.8
Hrs. 0
1
2
3
4
5
6
7
8
160 cc.N/6.35 HCl into jugular vein
P.R.
←—Light ether—→
pH tissues
pH blood
Died after 26 hrs.

CHART 1.

For the purpose of the foregoing observations the animals were stained with phenol red only after the injection of acid had proceeded for some time; since otherwise the staining did not last long enough to cover the entire later period of study. The plan was now adopted of giving smaller amounts of the indicator with repetitions of the dose at more frequent intervals. Tests directed to the point had shown that the intravenous injection of 10 cc. of 4 per cent phenol red, brought to pH 7.4, exerted not the slightest effect on the reaction of the blood. To expose the jugular vein novocaine was employed, without precautions for sterility. After the injection was finished the neck incision was sutured and the animal placed by itself in a metabolism cage where it could be carefully watched. Abstracts of two protocols will be given.

Experiment VI.—(See Chart 2.) Male white rabbit (No. 6) of 1690 gm. 9.30 a.m.—*First blood specimens taken.* 9.37—First injection of phenol red. 10.05—Rabbit coloring rapidly and evenly. 10.22.—*Surface hue* is *old rose;* by wedge method pH 7.5. 10.30—*Second blood specimens taken.* 11.29—*Injection of* N/*6.35 HCl begun* at rate of 5 cc. every 3 minutes. 12.06 p.m.—65 cc. in. Animal completely decolorized. 12.35—102 cc. in. Pause for injection of phenol red, 10 cc., by way of the jugular cannula, between 12.36 and 12.39 p.m. Injection of acid now renewed at a slower rate. 12.56—115 cc. of acid has been injected. The animal is everywhere *jasper red.* 2.29—The general color is *coral red* but with carroty areas here and there. *Injection terminated and animal removed from pad.* It has received 170 cc. of acid in all. 2.50—*Third blood specimens taken.* Animal O. K. 3.00—*Surface hue* between *coral red* and *carnelian red;* by wedge method pH 7.2. 4.33—*Fourth blood specimens taken.* 4.39-40—Injection of phenol red, 5 cc., into an ear vein. 5.13—*Surface hue* by wedge method pH 7.3−. 5.18—*Fifth blood specimens taken.* 5.41—*Surface hue* by wedge method just about pH 7.3 (artificial light).

During the latter part of the period of injection the respiratory amplitude was greatly increased. The animal remained quiet throughout, however. Afterwards it voided large quantities of urine colored orange-red with phthalein. During the night it escaped from the metabolism cage, and some urine may have been lost. Next morning the respirations were still as exaggerated as on the previous evening, and the animal was weak and apathetic.

Second Day, 9.55 a.m.—*Sixth blood specimen taken.* 10.08—Injection of phenol red, 5 cc. into an ear vein. 10.20—The rabbit has colored but poorly, yet there is no evident outlying acidosis. 10.39—The coloration is diffuse; *surface hue* by wedge method, pH 7.3+. 11.18—*Seventh blood specimen taken.* 11.33—*Surface hue* by wedge method, pH 7.3. Respirations still exaggerated. 12.07—There is

a sudden commotion in the cage, and the rabbit previously quiet, is found in opisthotonos. It died at 12.10.

The autopsy showed atelectasis of the upper lobes of both lungs, and in the right psoas muscle a contracted blood clot apparently about 24 hours old with a volume of about 10 cc.

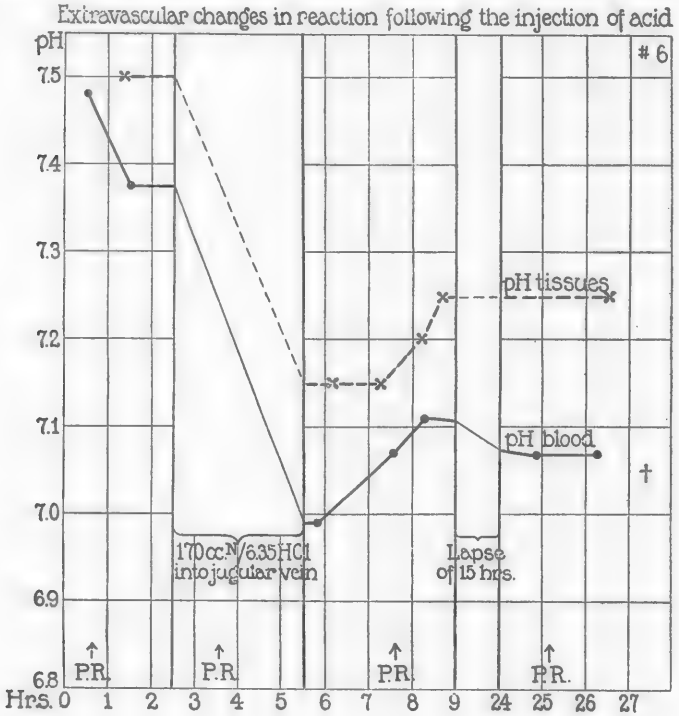

CHART 2.

The bladder had been emptied by catheterization at the beginning of the experiment. From then until death 184 cc. of urine was secreted, an amount somewhat larger than the total of acid solution injected. The rabbit had free access to water during the night and to food but appeared not to have partaken of either. The amount of circulating hemoglobin fell from 78 per cent to 58 per cent (Newcomer), and the red cells from 6,590,000 to 3,490,000 per c.mm. in the first 24 hours. A part of the blood change is attributable to the hemorrhage into the muscle.

In this experiment the maximum quantity of acid was given that could be borne. The reaction of the blood was altered from pH 7.4 to pH 7.0, and during the next 24 hours it scarcely changed (Chart 2). The surface color, recorded in terms of pH, gave readings that were regularly further on the alkaline side than those of the blood; but, as has already been emphasized, these readings cannot under the conditions be taken as expressive of the actual pH prevailing. They have merely a relative value as indicative of the extravascular condition. The change in this would appear to have been not quite so great as in the blood, the apparent pH falling from about pH 7.5 to pH 7.2. The later, slight recovery in the blood was duplicated, however, by an extravascular recovery of equal magnitude.

The death of the animal cannot be attributed directly to the acidosis because of the lesions found at autopsy. The question comes up, indeed, as to whether the persistence of the acidosis may not have been due to these latter. That it was not, the following experiment shows.

Experiment VII.—Male rabbit (No. 7) of 1650 gm. Jugular bared under local anesthesia with novocaine. 9.42—*First blood specimens taken.* 9.50½-52—Injection into an ear vein of 4½ cc. phenol red. 10.22—*Surface hue* between *old rose* and *eugenia red;* by wedge method, pH 7.5. 10.53—*Second blood specimens taken.* 11.02—*Surface hue* by wedge method pH 7.5. 11.46—*Injection of* N/6.35 *HCl begun.* 12.36 p.m.—59 cc. has been injected. 1.13—Injection of 5 cc. phenol red by way of the cannula in the jugular. The animal colored evenly and quickly, *jasper red.* 1.46 p.m.—132 cc. of acid in. *Surface hue* between *light jasper red* and *coral red.* 2.02 p.m.—142 cc. of acid in. Rabbit is restless; breathing greatly exaggerated. 2.19—Animal nearly decolorized. 2.24 p.m.—149 cc. in. 2.24-26½—10 cc. of phenol red injected into jugular. A rapid and deep staining developed. 3.01 p.m.—158½ cc. of acid has been run in; *injection terminated* and incision sewed up. 3.10—*Surface hue* between *coral red* and *jasper red.* 3.11—Rabbit removed from board. It is alert but stands weakly, and has a notable air hunger. 3.26—In excellent condition, though still weak and with air hunger. *Surface hue* now slightly darker than *carrot red.* 3.43—*Surface hue* by wedge method between pH 7.1 and pH 7.2. 3.52—*Third blood specimens taken.* 4.00—*Surface hue* again between pH 7.1 and pH 7.2. 4.51—*Surface hue* just above pH 7.1. 4.57—*Fourth blood specimens taken.* 5.03—*Surface hue* between pH 7.1 and 7.2. 5.20—Decolorizing. Left overnight, with water but no food. Eager to eat cabbage shown it.

Second Day. 9.35 a.m.—Animal in excellent condition except for weakness of fore leg on side of jugular injection. Breathing still exaggerated. 9.44—*Fifth*

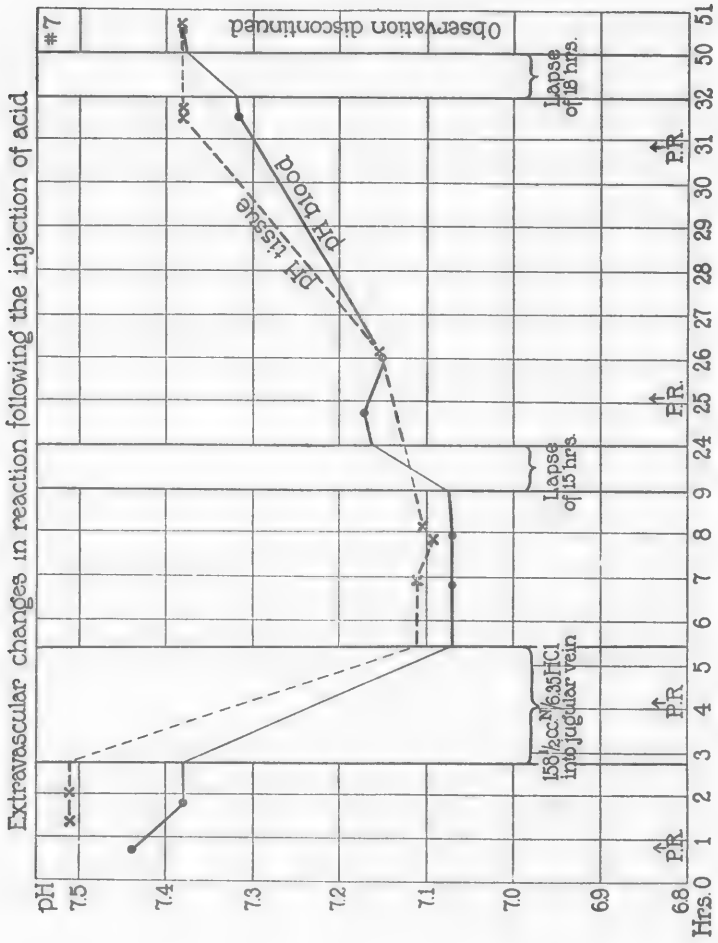
Extravascular changes in reaction following the injection of acid
#7
Observation discontinued
pH tissue
pH blood
Lapse of 18 hrs.
Lapse of 15 hrs.
158 c.c. N/6.35 HCl into jugular vein
P.R.
pH
7.5
7.4
7.3
7.2
7.1
7.0
6.9
6.8
Hrs. 0 1 2 3 4 5 6 7 8 9 24 25 26 27 28 29 30 31 32 50 51

CHART 3.

blood specimens taken. 10.04-06—7 cc. of phenol red injected into an ear vein. The staining that followed was rapid, even, and deep; *surface hue* by wedge method pH 7.3. 10.41—*Surface hue,* by wedge, just below pH 7.3. 11.00—*Sixth blood specimens taken.* 11.04—*Surface hue* by wedge, pH 7.2. 11.09—Color is between *light jasper red* and *light coral red,* slightly darker than either. 11.32—Given cabbage which it eagerly attacks. 2.15—Has eaten largely; condition excellent though right fore leg is still dragged. 3.53—Injected with 7 cc. of phenol red. 4.14—General color *jasper red.* 4.32—*Surface hue* by wedge, pH 7.4. 4.35—*Seventh blood specimens taken.* Still some air hunger on exertion. 4.42—*Surface hue* pH 7.4. 6.13—Left overnight with food. General condition as before.

Third Day. 10.24 a.m.—Has again eaten largely. Injected with 7 cc. phenol red. 10.35—Given more cabbage; eating. 11.07—*Surface hue* by wedge pH 7.4, and again pH 7.4 at 11.24. 11.28—*Eighth blood specimens taken. Surface hue* by wedge pH 7.4. Decolorizing; color is *old rose.* 12.27—Not yet fully decolorized; observations terminated.

Fourth Day. Weight 1500 gm.

Fifth Day. The tissues of the neck are indurated about the old wound. Animal killed with chloroform. The general condition of the organs is excellent save in in the case of the kidneys, the renal cortex appearing somewhat swollen and paler than normal (parenchymatous degeneration?).

The bladder had been emptied by catheterization prior to the injection of acid, and during the 19 hours thereafter 223 cc. of urine heavily colored with phthalein was voided. During this period no food had been given.

The hemoglobin dropped from 75 per cent prior to the experiment to 58 per cent on the next morning, and the number of red cells from 5,460,000 to 4,430,000. In the afternoon the hemoglobin percentage was 65 and the number of red cells 4,750,000. On the 2nd day the figures were the same. For the determinations and those of Experiment VI we are indebted to Dr. Charles A. Doan.

In this experiment (Chart 3) the alteration in the surface color, as recorded in terms of pH, was slightly greater than that found in the blood, whereas in the preceding one it had been slightly less. The correspondence between the later extravascular changes, indicated by the surface hue, and the intravascular ones was exceedingly close. The observer following the alterations in the blood did not know of those in the surface hue. The variations in pH were almost identically the same in both, and the same very gradual recovery took place until food (cabbage) was given, when the acidosis rapidly lessened, almost disappearing within a few hours. How long recovery might have taken had no materials to combat the acidosis been supplied by mouth is problematic.

The amount of urine voided by the animal during the first 19 hours after the acid injection (223 cc.) considerably exceeded the fluid bulk introduced in this way (158½ cc.).

The diminution in the amount of blood in circulation attested by the lessening in hemoglobin and in the number of circulating red cells is to be attributed in no small part to the repeated bleedings incident to the observations on the blood reaction. The remaining loss was doubtless due to an action of the hydrochloric acid solution on such red cells as came into direct contact with it during the injection period.

Significance of the Extravascular Changes.

The alterations in the surface color that took place during the foregoing experiments might be attributed wholly to changes in the reaction of the stained extravascular fluid did not incidental observations show that the hue of the tissues themselves was altered. We have already described the method whereby it is possible to determine the hue of connective tissue *in situ*. Oiled folds of skin of the vitally stained rabbit when viewed between slides by transmitted light are ordinarily pink even when the fluid has been driven out of them by pressure upon the slides. After the injection of acid they appeared orange-pink until pressure was made when they were seen to be of a clear orange-yellow, far more acid, judging from the color, than the body surface as viewed by reflected light. The conjunctiva likewise became orange, and the sclera palely so. The cartilage in the least vascular portions of the ears of vitally stained rabbits is pink ordinarily. But after the acid injection it was a clear orange-yellow. In proportion as the general acidosis was recovered from, so too did all of the tissues mentioned alter from orange to pink. On the inside of the ear where the cartilage is covered with merely a film of adherent skin its color can be readily followed by reflected light; and the observation was made that at times when the alteration in the hue of the body surface generally had progressed no further than *coral red* the cartilage had become a clear orange. The finding would suggest that despite the relative alkalinity of normal rabbit cartilage as compared with that of the rat or mouse, an alkalinity finding expression in a pink staining with phenol red,

this tissue is still not so alkaline as the connective tissue, which in turn is less alkaline than the blood (11). The pH of normal rabbit blood is almost identical with that of the rat (12).

DISCUSSION.

The findings show that when changes in the reaction of rabbit blood are induced with hydrochloric acid extravascular changes of approximately the same magnitude occur. No dissections were made to inspect the interior organs; but from previous experiments on rats it seems certain that conditions of blood acidosis or alkalosis leading to a change in the color of connective tissue and cartilage will also lead to one in the color of the tendons, whereas the hue of liver, pancreas, and lymph nodes will be unaffected even when the blood change is so great as to lead to death (13). There is every reason to suppose that the same holds true in the case of rabbits, and that by following the visible alterations in the connective tissue and cartilage one can obtain an index to much of what would be disclosed by dissection under oil.

The failure of the extravascular reaction to alter more than does that of the blood would at first sight seem surprising. For, with a lessened gradient between tissues and blood, one might suppose that there would be some heaping up of the acid products of metabolic activity. Perhaps such a heaping up and not a passage of H ions from the blood is actually one of the causes of the extravascular acidosis. Whenever the circulation is interfered with, as during ether and urethane anesthesia, a heaping up of acid metabolites certainly does occur, with result in an "outlying acidosis" independent of that due to the injected acid. One is reminded in this connection of Yandell Henderson's statement that most patients and animals under general anesthesia for more than an hour are in the first stage of shock (14). Our experiments prove that under the conditions of such anesthesia an abnormal local reaction develops within some of the tissues which may well prove detrimental to the organism as a whole.

The demonstration that a widespread extravascular acidosis occurs when there is a marked blood acidosis, and that it is tolerated for

long periods has a bearing upon the problem of shock. For many years now the relationship between the functional state of the small blood vessels and the reaction of their milieu has been debated; and many have supposed that a vascular dilatation is caused by acidosis and that such a dilatation may be responsible for shock through the changes in the distribution of blood that it entails. The British Commission for the investigation of surgical shock could not produce it by injecting acid into the circulation (15); but they were unable to bring proof that in altering the condition of the blood they also altered the reaction outside of the lumen of the vessels. That extravascular changes go hand in hand with those taking place in the blood has been demonstrated in the course of the present work. Yet not the least evidence of shock was to be seen in our animals though the blood reaction was diminished to the limit compatible with life and the extravascular reaction of large regions was proportionately affected. Though the connective tissue was markedly acidotic, as shown by its color during the periods of staining, not the least vascular dilatation could be observed in it through the shaved and oiled skin. In the intervals of decolorization between the injections of phenol red there was good opportunity to make observations on the latter point.

The quantities of fluid voided by our animals during the many hours that the tissue acidosis endured regularly exceeded the very large amounts introduced with the acid. These animals had access to water but were given no food, and they certainly did not drink largely. There was not the least sign of a fluid retention in the acidotic tissues. In a previous paper (16) one of us has demonstrated that local acidosis and edema sometimes occur together but without any relationship of cause and effect. The present findings afford another example of the phenomenon.

SUMMARY.

The changes in blood reaction caused by the injection into a vein of a weak solution of hydrochloric acid are accompanied by extravascular changes of similar magnitude within the subcutaneous tissue. Under the conditions of prolonged general anesthesia with

ether or urethane the circulation to this tissue is so interfered with that an "outlying acidosis" may develop in addition to the acidosis immediately consequent on the blood state. Even under the best of circumstances the extravascular acidosis induced with hydrochloric acid affects not merely the tissue fluid but the reaction of the tissue itself.

Rabbits in which a widespread extravascular acidosis has been produced, together with a blood acidosis as severe as is compatible with life, remain in good condition during the relatively long period over which this state of affairs persists. There is at no time any sign of capillary dilatation, though the vessels are bathed in relatively acid fluid, and none of shock. No edema develops in the acidotic tissues, and the animals void large amounts of urine. The tissue acidosis lessens *pari passu* with that of the blood.

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THE RELATIVE REACTION WITHIN LIVING MAMMALIAN TISSUES.

IX. On the Tissue Reaction as Influenced by Inhalations of CO_2 and by Overbreathing.

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(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

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In preceding papers from this laboratory the influence upon the tissue reaction of injections into the circulation of weak solutions of acid and alkali has been recorded, and the relation existing between the blood changes and those in the tissues during the course of an acidosis induced in this way has been described (1). We have now sought to determine the effect upon the tissues of breathing high concentrations of carbon dioxide, and of the overventilation that results from forced respiration.

The Inhalation Apparatus.

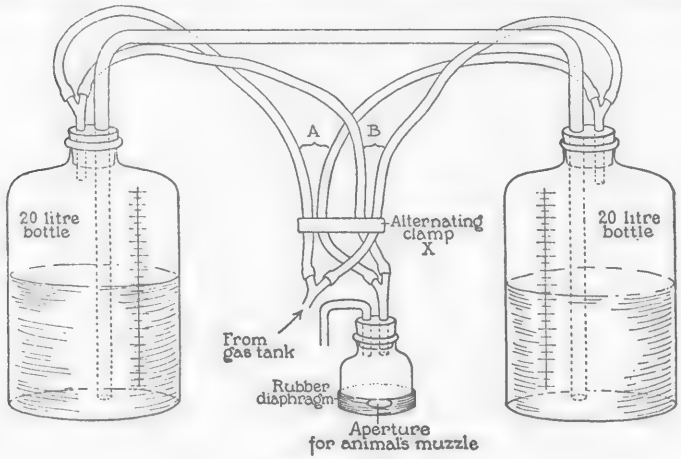
White rabbits were used. The general technic of vital staining with phenol red and of appraising the reaction of the blood and the changes in the color of the body surface have already been detailed (2). The buffer solutions used in the wedges and to determine the reaction of the blood differed slightly in pH, as determined with the potentiometer, from the calculated values. In charting the results corrections have been made for the differences, whereas in the protocols the figures as originally read off are recorded.

A special apparatus was necessary, for the purpose of the inhalations of carbon dioxide. It is portrayed in Text-fig. 1.

The gas from the storage cylinder escaped into one or the other of two 20 litre bottles graduated in half litres and about half full of water weakly acidulated with hydrochloric acid. The pressure under which the gas passed into the one bottle was sufficient to force water over into the other through the large tube connecting the two, and the only way of escape for the carbon dioxide already in this other was through a tube which led to the inhalation mask fixed upon the animal's muzzle. By means of a special snap clamp (X) acting, now on the tubes A, and again on B, the direction of flow from the storage cylinder could be alternated to

the bottles at will without interruption of it. An observer with a stop-watch controlled the speed at which gas passed to the inhalation mask. One litre per minute was the ordinary rate.

The mask was made out of a short broad bottle from which the bottom had been cut. Across the opening thus provided a diaphragm of rubber was stretched with a hole in the center for the rabbit's muzzle. There was a continuous current of gas through the bottle, and the space in it was small, to prevent rebreathing. The tube through which the gas escaped was long in order that there might be no sucking back of air into the mask on inspiration. To ensure a snug fit of the rubber diaphragm all hair was removed from the animal's muzzle with a sodium sulfide solution. The mask was held in place by a strip of adhesive tape passing



TEXT-FIG. 1.

around the back of the head, and there was never any leakage between it and the skin. Care was taken not to interfere with the circulation to the ears.

Five experiments were performed with a mixture containing 21.08 per cent of CO_2, and approximately 22.09 per cent O, and 56.83 per cent N. A single set of observations were made with 37 per cent CO_2, 22 per cent O and 41 per cent N. For the analyses of the gas mixtures, as supplied in pressure cylinders, we are indebted to the kindness of Dr. C. A. L. Binger.

In four of the five experiments just referred to, urethane was employed as anesthetic. The blood specimens for the pH determinations were procured from a

femoral vein. In the case of the remaining animal, which received no general anesthetic the small incision necessary to bare the vein was made after infiltration of the tissues with novocaine. To aspirate blood Luer needles bent at a reentrant angle were used, as in the previous work. It was essential that the circulation of the leg furnishing the blood should not be interfered with, so no thong or other tie was placed about it. The vein could be punctured again and again in the groin without any important bleeding or thrombosis, if the needle was thrust slantingly through its sheath and pressure applied for a few moments after the aspiration.

In several of the experiments a minimum dose of urethane was employed and a far better respiratory response to the acidosis was obtained than in the animals more heavily anesthetized by its means and given hydrochloric acid (3).

The Acidosis on Breathing 21 Per Cent of CO_2.

The protocol of *Experiment 1* need not be given since the facts are better illustrated by the later work.

Experiment 2.—Male rabbit No. 2, of 1650 gm., given 3.3 cc. of 50 per cent urethane subcutaneously into the neck at 9.20 a.m., and 1 cc. more at 10.35, into the peritoneal cavity. 11.00—10 cc. of 4 per cent phenol red isotonic with 0.9 per cent NaCl and at pH 7.4 was injected into an ear vein. 11.50—Rabbit for some time on the warm pad. *Surface hue* slightly less purple than *eugenia red* (Ridgway); by wedge method between pH 7.5 and 7.6. Skin folds compressed between slides are pink by transmitted light with faintest yellow admixture; ear cartilage pale pink. 11.54—*First blood specimens taken.* 12.00—*Surface hue* between pH 7.5 and 7.6.

12.01—*Mask adjusted and gas run in.* The respirations at once mounted from 40 to 88 per minute, much exaggerated; but almost as rapidly they lessened in frequency again.

12.05—*The surface hue* generally has become yellower, between *jasper red* and *light jasper red.* No indication of spotting. 12.07—Respirations 56 per minute, regular. 12.10—*Surface hue* yellower than *jasper red.* Skin folds orange-yellow. 12.12—*Surface hue coral red.* 12.41—*Surface hue light coral red.* Skin folds pronouncedly orange-yellow; ear cartilage light yellow. 12.48—*Surface hue* by wedge method, pH 7.2. 12.51—*Second blood specimens taken.* 12.55—*Surface hue* by wedge, pH 7.2. 12.56—*Surface hue* still *light coral red.* During this long inhalation period the respirations have been much exaggerated but never stormy. Their rate has lessened from 56 to 48 per minute.

12.57—*Mask removed.* The respirations at once became much less ample, 52 per minute. Practically at once, too, the *surface hue* became pinker, the change being very definite by 12.59.

1.01—Animal stirred, as it had not during the inhalations. 1.03—Skin fold much less yellow. 1.08—*Surface hue* now approaches *old rose*. 1.12—*Surface hue* by wedge, pH 7.4. 1.14—Ear cartilage more pink than yellow now, while skin fold is as pink as before the inhalations. 1.19—*Surface hue* between *jasper pink* and *old rose*, pH 7.4 by wedge. 1.26—*Third blood specimens taken.* 1.30—*Surface hue* by wedge pH 7.4−; between *light jasper red* and *jasper pink*. Observations discontinued. The respirations varied between 52 and 40 after the mask was off; they were quiet.

Experiment 3.—Performed on afternoon of same day as Experiment 2 and upon the same animal, which was still unconscious owing to the urethane. 2.52—Given 8 cc. of phenol red into the same ear vein as before. 3.17—*Surface hue* slightly purpler and deeper than *jasper red*, by wedge method pH 7.5+. 3.19—*First blood specimens taken.* Ear cartilage is light pink; skin folds, compressed, are pink, with faintest yellow tinge. 3.23—*Surface hue* by wedge, pH 7.5+.

3.28—*Mask adjusted and inhalations begun.* The respirations, which had averaged 48 per minute, quickened greatly at once but soon slowed again, to 62, much exaggerated.

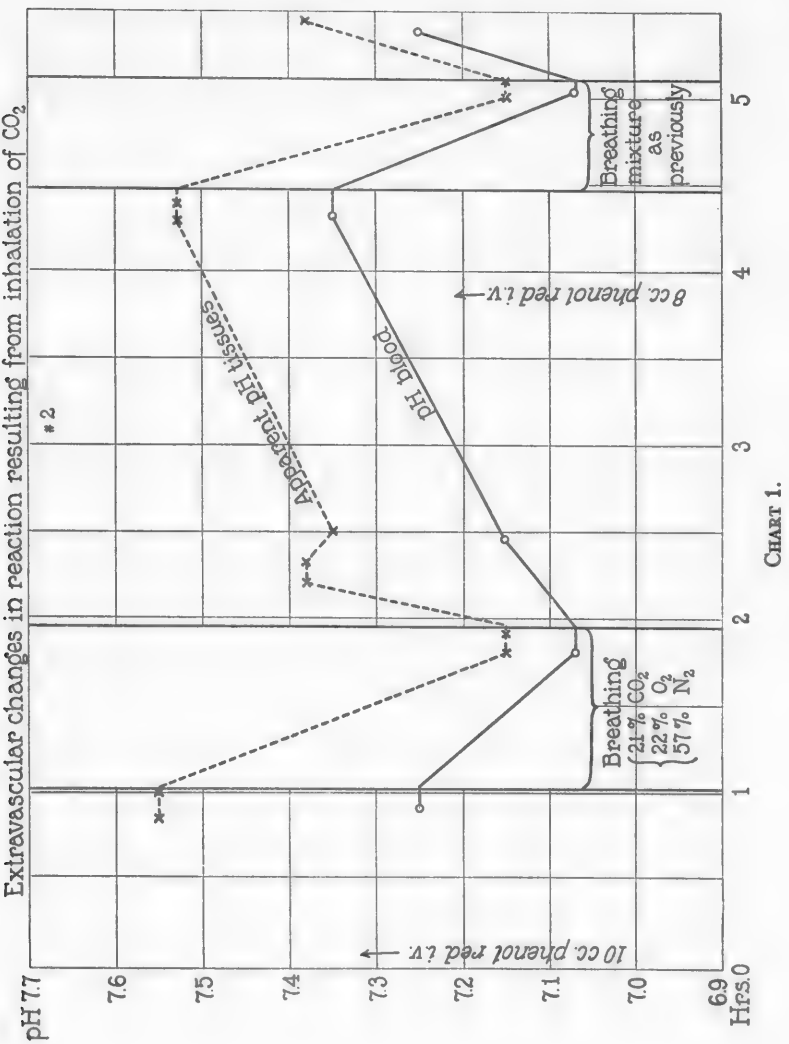
3.32—*Surface hue* yellower. 3.33—Skin folds much yellower. Some surface patching of color. 3.35—The general color is *jasper red*, with some *coral red* patching over chest and abdomen. The patches are irregular, only a few centimeters across. They have the general character of those seen in outlying acidosis. 3.43—The *coral red* patches have become confluent, everywhere driving out the rosy hue. A skin fold is bright orange-yellow but the color of the ear cartilage has not changed. 3.53—*Surface hue* pH 7.2+ by wedge method. There is a definite yellowing of the ear cartilage. The circulation in the ear is excellent. 4.00—*Surface hue* still that of pH 7.2+. 4.02—*Second blood specimens taken.* 4.07—*Surface hue* between *coral red* and *light coral red;* by wedge method pH 7.2+. Ear cartilage yellow. During the inhalations the respirations gradually slowed from 62 to 44 per minute, markedly exaggerated always.

4.08—*Mask removed.* Immediately the breathing became stormy and quick for a few moments, but then quieted and slowed to a rate of 48 per minute and gradually, by 4.30, to 32 per minute. The color of the animal at once began to change back to the normal, being definitely more ruddy by 4.11. The change was generalized.

4.13—*Surface hue* between *jasper red* and *light jasper red;* skin fold pink, with but a slight admixture of yellow; ear cartilage still yellowish. 4.19—*Surface hue* between *light jasper red* and *old rose*. 4.25—*Third blood specimens taken.* 4.28—*Surface hue* by wedge, pH 7.4. 4.30—Animal stirring slightly.

Observations discontinued. 50 cc. of warm water given by stomach tube. Next morning the rabbit was in excellent condition, eating.

The buffer solutions used in the wedges and to determine the reaction of the blood differed slightly, on test with the potentiometer, from the calculated pH values. Corrections have been made for the differences in charting the results, whereas in the protocols the figures originally read off are given.


Extravascular changes in reaction resulting from inhalation of CO_2
pH 7.7
7.6
7.5
7.4
7.3
7.2
7.1
7.0
6.9
Hrs. 0
1
2
3
4
5
#2
10 cc. phenol red i.v.
8 cc. phenol red i.v.
Breathing { 21 % CO_2 22 % O_2 57 % N_2
Breathing mixture as previously
Arterial blood pH
Subcutaneous tissue pH
Chart 1.

As the protocols and the chart show (Chart 1) there was in these experiments not only a great alteration in the reaction of the blood but one in the extravascular reaction as well, when the animal inhaled a gas mixture containing 21 per cent CO_2 and the normal quantity of oxygen. The changes in the surface color indicative of an increasing acidity began practically at once, and, progressing rapidly, reached a maximum some little time before the inhalations were discontinued. When this had been done an immediate return toward the normal took place, at first rapid, then more gradual. In Experiment 3 there was, as the general acidosis developed, a patching which resembled that of outlying acidosis.

Experiment 4.—Male rabbit No. 4 of 1700 gm. given 4.5 cc. of 50 per cent urethane into the peritoneal cavity at 9.20 a.m. and 10 cc. of 4 per cent phenol red into an ear vein at 10.04. 10.25—Staining is even and deep; animal on warm pad, deeply anesthetized. 10.48—*Surface hue* not quite so purple as *eugenia red;* by wedge method at pH 7.5. 10.54—*First blood specimens taken.* 10.57—*Surface hue* still at pH 7.5. A skin fold is pink with slightest tinge of yellow; ear cartilage pink.

11.08—*Inhalations begun.* Immediately the respirations increased in rate and amplitude.

11.12—*Surface hue* is yellower. 11.14—*Surface hue* now *jasper red* save for a longitudinal streak about 4 cm. wide extending from ensiform nearly to symphysis, which is still purply red. 11.15—Skin fold more yellow. Respirations still markedly exaggerated but of nearly the same rate as before the inhalations were begun, 48 as compared with a previous 44 to 46 per minute. The rate altered practically not at all from now until the mask was removed. 11.23—Dubious yellowing of ear cartilage. Good circulation in the ear. 11.29—*Surface hue* between *jasper red* and *coral red.* 11.33—Skin folds are much more yellow; and so too is the ear cartilage. 11.40—*Surface hue* generally is between *coral red* and *light coral red.* The median streak of differing color has disappeared. 11.48—Circulation to ear is excellent yet cartilage is orange-yellow, as also are the skin folds. 11.52—*Surface hue* by wedge method pH 7.2+. *Second blood specimens taken.* 11.59—*Surface hue* at pH 7.2; yellower than *coral red.* Color of skin folds and cartilage orange-yellow.

12.00—*Mask removed.* Respirations temporarily increased in amplitude and frequency, being 60 to the minute at 12.01 but soon becoming quiet and slowing to 40. Practically no change took place in the *surface hue* for a long time, however. At 12.37 it was still between *coral red* and *light coral red;* and at 12.40, by wedge method, at pH 7.2.

12.46—*Third blood specimens taken.* 12.49—*Surface hue* by wedge pH 7.2. 2.00 p.m.—Rabbit still deeply unconscious, an even *light jasper red.* Skin surface

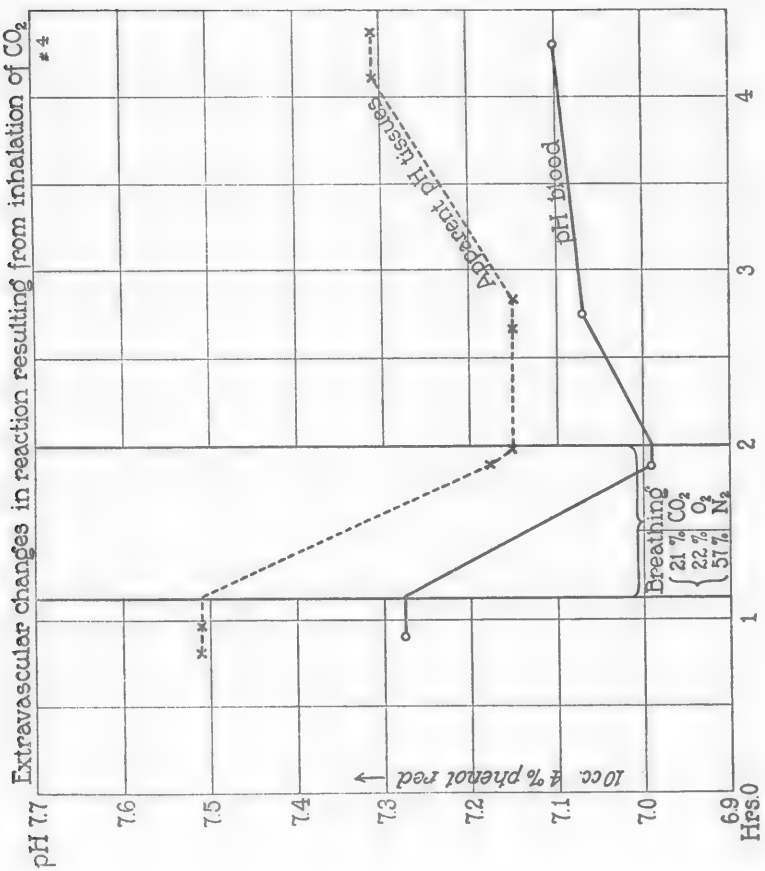

Extravascular changes in reaction resulting from inhalation of CO_2
#4
pH 7.7
7.6
7.5
7.4
7.3
7.2
7.1
7.0
6.9
Hrs. 0
1
2
3
4
10 cc. 4 % phenol red
Apparent pH values
pH blood
Breathing
21 % CO_2
22 % O_2
57 % N_2

CHART 2.

is warm. 2.07—*Surface hue* between pH 7.3 and pH 7.4. Skin folds still much yellower than normal. Ear cartilage has decolorized too far for appraisal of the hue. 2.18—*Fourth blood specimens taken.* 2.24—*Surface hue* by wedge between pH 7.3 and pH 7.4. 2.25—*Surface hue* between *light jasper red* and *old rose.* Skin fold still is yellower than "normal." Animal has not stirred at all. 5.00—Color still a medium light pink. Animal deeply unconscious. Observations discontinued. 50 cc. of warm water was given by gavage and next day the rabbit was in excellent condition.

The injection of urethane into the peritoneal cavity resulted in a deeper anesthesia than when the same dose was given subcutaneously (Experiments 2 and 3). The rabbit never moved during the observations, and the staining persisted for an unusually long period. But more important was the persistence of acidosis after the mask had been taken off (Chart 2). Comment has already been made (4) on the action of urethane to lessen or prevent the compensatory respiratory response that is usually called forth by an acidosis. As result of this occurrence the acid products of metabolism tend to heap up within the organism to a greater extent than would follow merely from the introduction of acid from without. In the present case the extravascular acidosis indicated by the surface hue was not unusually marked; and hence one can scarcely attribute its persistence to an unusual accumulation of acid metabolites. Doubtless the depression of the respiratory activities persisted after the mask had been taken off.

As a check upon the findings observations were now made in the absence of a general anesthetic.

Experiment 5.—Male rabbit No. 5 of 1500 gm. 10.25–30 a.m.—10 cc. of phenol red given into an ear vein. The animal at once began to color up rapidly and evenly. It was placed on the warm pad and a femoral vein bared with the aid of novocaine. 11.02—*Surface hue* slightly less purple than *eugenia red;* by wedge method at pH 7.6. 11.05—*First blood specimens taken.* The animal is quiet. 11.10—Skin flaps are pink, with the slightest yellow admixture. The cartilage of the ear is deep pink. 11.14—*Surface hue* by wedge still at pH 7.6.

11.15—*Inhalations begun.* The breathing at once became rapid and stormy but the animal did not struggle then or later. The respiratory rate rose abruptly from 80 to 110 but gradually fell again, reaching 78 at the end of the inhalation period. Throughout it the breathing was greatly exaggerated.

11.17—*Surface hue* rapidly losing its purple quality. Some linear razor marks heretofore not visible are now rendered suddenly prominent by an orange border

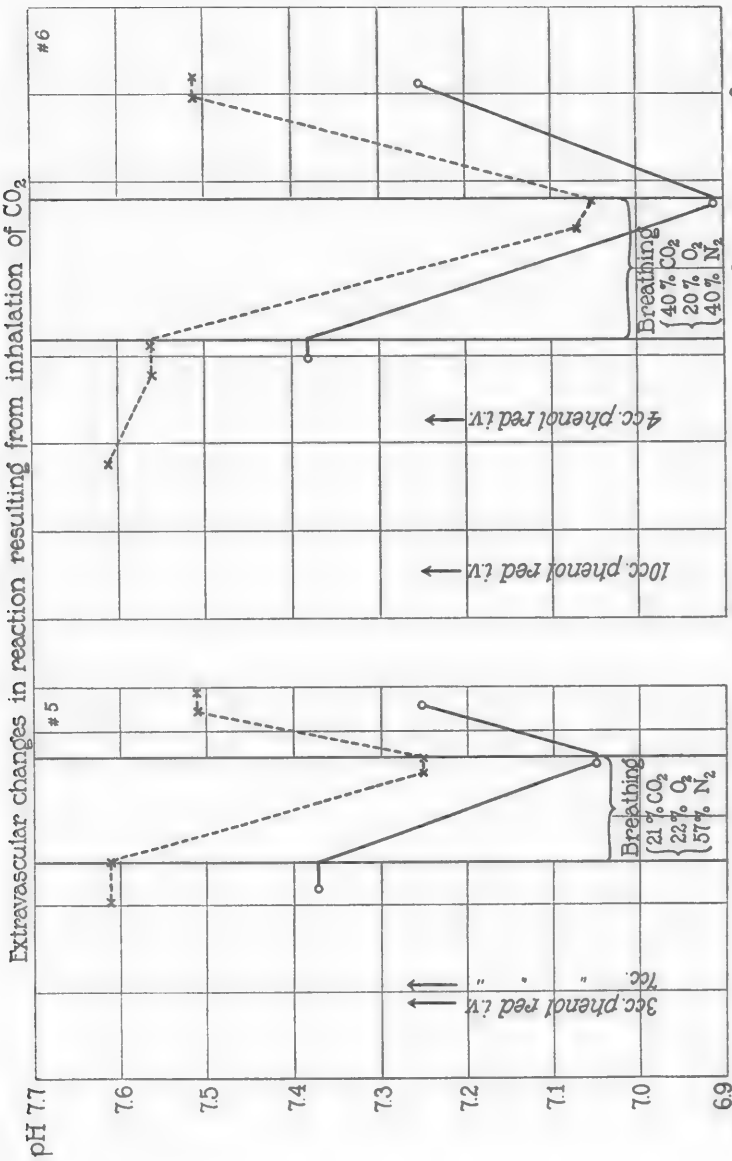

Extravascular changes in reaction resulting from inhalation of CO_2
pH 7.7
7.6
7.5
7.4
7.3
7.2
7.1
7.0
6.9
Hrs. 0
1
2
#5
3cc. phenol red i.v.
7cc. " " "
Breathing
21 % CO_2
22 % O_2
57 % N_2
#6
10cc. phenol red i.v.
4cc. phenol red i.v.
Breathing
40 % CO_2
20 % O_2
40 % N_2
3
Apparent pH tissues
pH blood

CHART 3.

to either side of them. 11.20—*Surface hue* nearing *jasper red.* 11.23—There is a pronounced spotting over the abdomen, of many orange-red patches about 1 cm. in diameter, scattered on a more ruddy background. The veins are nowhere turgid. 11.25—The patching has disappeared and the acidotic hue is confluent save for a broad streak from ensiform to pubis which is still purply red. The streak corresponds with the region where a poorly vascularized aponeurosis underlies the skin. The skin flaps are a marked orange-yellow and so too is the ear cartilage. The circulation in the ear continues excellent. 11.29—*Surface hue* between *coral red* and *light coral red.* The inside of the ear, where a bloodless cartilage is but thinly overlain with skin, is no longer pink by reflected light but yellow. The scleral conjunctiva has turned ruddy orange. There is an excellent conjunctival reflex and the animal appears conscious. 11.38—*Surface hue* slightly lighter than *coral red;* the median streak of more purply red has almost completely merged in the general hue. Skin flaps are clear orange-yellow; ear cartilage orange-yellow. 11.44—*Surface hue* at pH 7.3 by wedge method. 11.49—*Second blood specimens taken.* 11.50—*Surface hue* at pH 7.3.

11.50—*Mask removed.* Immediately the respirations became even more ample than before and the animal appeared excited. The superficial veins over the abdomen and chest became greatly engorged even in the finer ramifications.

11.52—*Surface hue* redder and conjunctiva has become pink again; but skin flaps are still orange-yellow. 11.54—Animal has now quieted and so too have its respirations. 11.57—*Surface hue* between *eugenia red* and *old rose.* The venous congestion continues. 11.59—The skin flaps have returned to the usual pink with faintest admixture of yellow; the ear cartilage is much pinker. 12.06—*Surface hue* as at 11.57; by wedge method at pH 7.5. 12.08—*Third blood specimens taken.* 12.15—The animal has struggled at intervals but remains quiet during repair of the groin incision. The veins over chest and abdomen are still notably congested. 12.17—Circulation in the ears is greatly cut down, and the cartilage is still somewhat yellow both by transmitted and reflected light. 2.40—Animal in excellent condition, very light pink. 3.05—Animal decolorized except for the ears which show a light pink cartilage. The superficial veins are still engorged. Observations discontinued. The animal was let up, when it behaved normally.

The results of this experiment (Chart 3) corresponded in general with those of the preceding ones. The tissue acidosis did not become so great, however, and recovery from it was more prompt, though not quite complete when the observations were terminated.

The Acidosis on Breathing 37 Per Cent of CO_2.

During the latter part of the inhalation periods of the foregoing experiments the surface hue changed but little. It was evident that the

limits had been reached of the extravascular acidosis that could be induced with 21 per cent CO_2. In order to obtain more marked changes resort was had to a mixture containing 37 per cent of this gas.

Experiment 6.—Male rabbit No. 6 of 1500 gm. No general anesthetic. At 10.16 a.m. 10 cc. of phenol red was given into an ear vein and a little later the animal was placed on the warm pad and a femoral vein bared with the aid of novocaine locally. 10.52—*Surface hue* slightly less purple than *eugenia red;* by wedge method at pH 7.6. 11.08—Beginning to decolorize, so 4 cc. more of phenol red given into same vein. 11.23—*Surface hue* slightly yellower than at 10.52; by wedge method between pH 7.5 and pH 7.6. 11.30—*First blood specimens taken.* 11.33—*Surface hue* slightly above pH 7.5. Skin flaps are pink with a trace of yellow, and ear cartilage also. The circulation in the ear is good.

11.35—*Mask on and inhalations begun.* The animal held its breath for about half a minute, then struggled, soon became quiet, and began to breathe regularly and very deeply. During the inhalation period the respiratory rate gradually lessened from 68 to 42 per minute.

11.38—*Surface hue* rapidly becoming yellower, nearing *jasper red* except along midline of abdomen, where as yet there is little change. 11.40—Ear cartilage much yellower. 11.41—Skin flaps already ruddy orange. 11.42—*Surface hue,* generally, *jasper red.* 11.51—Ear cartilage orange-yellow. 11.52—*Surface hue coral red.* 11.53—Animal appears to be completely unconscious; slight rhythmic movements of legs; good conjunctival reflex. 12.00—*Surface hue* about midway between *coral red* and *carnelian red.* 12.01—Skin flaps more orange. 12.05—Bloodless ear cartilage yellow by reflected light. 12.09—*Surface hue carnelian red.* 12.14—*Surface hue* by wedge method at pH 7.1. 12.22—*Second blood specimens taken.* 12.23—*Surface hue* slightly yellower than *carnelian red;* by wedge method pH 7.1 −.

After 12.00 n. the accessory muscles of the neck were called into play during the respirations. By 12.18 these were labored and less extensive. For fear that a gradual failure of ventilation might ensue and complicate the findings the mask was removed at 12.24. At that time there were 42 breaths to the minute. The color of the animal was still changing toward orange. Immediately that the mask was taken off the respirations became much shallower and the rate rose to 82 per minute. The rabbit, previously unconscious to all appearance, raised its head at 12.26 and struggled at 12.27. The ruddy color was now rapidly coming back.

12.28—*Surface hue* approaching *coral red.* 132 respirations per minute, shallow. 12.33—Rabbit quiet; 100 respirations per minute. *Surface hue* here *jasper red,* there *coral red.* 12.36—Conscious but quiet. 12.43—*Surface hue* only slightly yellower than *old rose.* 12.47—Ear cartilage still definitely yellower than ordinary. 12.48—Skin flaps have the normal pink hue. 12.58—*Surface hue* is *old rose;* by wedge method at pH 7.5. 1.03—*Third blood specimens taken.* 1.05—*Surface hue* by wedge, pH 7.5. Experiment discontinued. During its course the rabbit had

lost somewhat less than 5 cc. of blood by a slow escape from the femoral vein. When seen next morning it was in excellent condition.

The effect of breathing an atmosphere containing 37 per cent CO_2 with about the ordinary quantity of oxygen was to render the animal unconscious, and to induce a progressive acidosis (Chart 3). Toward the end of the inhalation period there were signs of respiratory failure; and complications from this source would doubtless have occurred had the mask not been taken off. The extravascular acidosis induced was more considerable than with 21 per cent CO_2 and apparently its limits were not reached. The acidosis of the blood became as pronounced as is ordinarily compatible with life, according to other investigators. Recovery was prompt but not quite complete during the brief period of observation.

Effects of Overventilation.

As a corollary the influence upon the tissue reaction of overbreathing was studied. It was already known that flaps of living and well vascularized connective tissue become more alkaline when exposed to air (5). So too does the peritoneal lining. With overbreathing sufficient to reduce considerably the carbon dioxide tension of the blood one would expect some change in the extravascular reaction. Such a change was obtained, a definite but not a marked one as would follow from the fact that the induced blood alkalosis was but slight. Six experiments were performed, upon rabbits.

Under general anesthesia, brought about with urethane in all save one instance, the animals were tracheotomized and a limb of a T-tube passed down nearly to the bronchial bifurcation and tied in place. In the exceptional case ether was used during the tracheotomy and the lips of the incision were swabbed with novocaine prior to discontinuance of the general anesthesia, at the time when the experiment proper was begun. The tube that formed the staff of the T was connected with the house suction in some instances and in others left open, while the free limb was connected with a motor-run mechanism devised by Dr. F. L. Gates, whereby air, separately warmed and moistened, was blown continuously or rhythmically into the lungs. The chest of some of the urethanized animals was opened by a bloodless incision down the middle of the sternum and a screw retractor inserted to expose the lungs. The blood specimens were taken from a femoral vein, and the surface hue appraised by the wedge method as usual.

Blowing air continuously for half an hour through a catheter with its opening down almost as far as the bifurcation of the bronchi was without effect on the surface hue of the animal stained with phenol red; and when the stream of air was cut off no apnea ensued. Filling and emptying the lungs by the alternate blowing in of air, and suction upon the tracheotomy tube with the house vacuum resulted in well defined changes but great care had to be taken to control the pressure relations, else pulmonary hemorrhage ensued. The best results were obtained in animals with relatively flexible thoracic walls which permitted of a large expansion and deflation of the lungs. It was found that when the chest was opened the animal developed the signs of a marked outlying acidosis despite the existence of an overventilation as proven by the apnea that ensued when the artificial respiration was stopped. In our opinion this acidosis resulted from a peripheral vascular constriction, secondary to and compensatory for, an interference with the circulation which was in turn traceable to an embarrassment of the heart. This last organ,—no longer provided with its usual orientation and supports,—collapsed upon itself at each deflation of the lungs.

The alterations in the reaction of the blood and tissues were never great; and the surface hue was more difficult to appraise in terms of pH than was the case in the acidosis experiments, because the colors were well toward the alkaline side of the range of phenol red. The colors could not always be recorded precisely in terms of Ridgway's nomenclature because the values associated with alkalosis differed somewhat from his scales. Two protocols will be given.

Experiment 7.—(*a*) Male rabbit No. 7 of 2075 gm. given 4 cc. of 50 per cent urethane into the peritoneal cavity at 12.02 and 10 cc. of phenol red into an ear vein at 1.08. A few whiffs of ether were administered during the tracheotomy, which had been accomplished by 1.27 p.m. 1.37—*Surface hue* between *eugenia red* and *jasper red* but deeper than either; by wedge method pH 7.5−. Skin flaps pink, with faintest yellow admixture.

1.40.—*Artificial respiration by pressure and suction begun.* The suction was sufficient to cause marked depression at the costochrondral junctions and the normal respiratory rhythm was easily overcome. 1.46−On intermitting briefly the artificial respiration an apnea ensued lasting 45 seconds. 2.00—*Surface hue* deeper than *eugenia red;* by wedge pH 7.6+. 2.01—Skin flaps deep purply red, no yellow.

2.23—*Discontinued artificial respirations. Surface hue* at this time just above pH 7.6 by wedge. Apnea for 70 seconds; then resumption of breathing at 44 respirations per minute. During the suction and compression the rate had varied between 28 and 32. 2.27—*Surface hue* returning toward *eugenia red.* 2.39—*Surface hue* between *eugenia red* and *jasper red.* 2.55—*Surface hue* less purple and slightly lighter than *eugenia red.* Skin flaps once again show a tinge of yellow in the prevailing pink.

(*b*) 3.00 p.m. of same day—*Surface hue* by wedge pH 7.5. 3.01—*First blood specimens taken.* 3.05—*Surface hue* by wedge pH 7.5−. Skin flaps colored as before. Respirations 42 per minute.

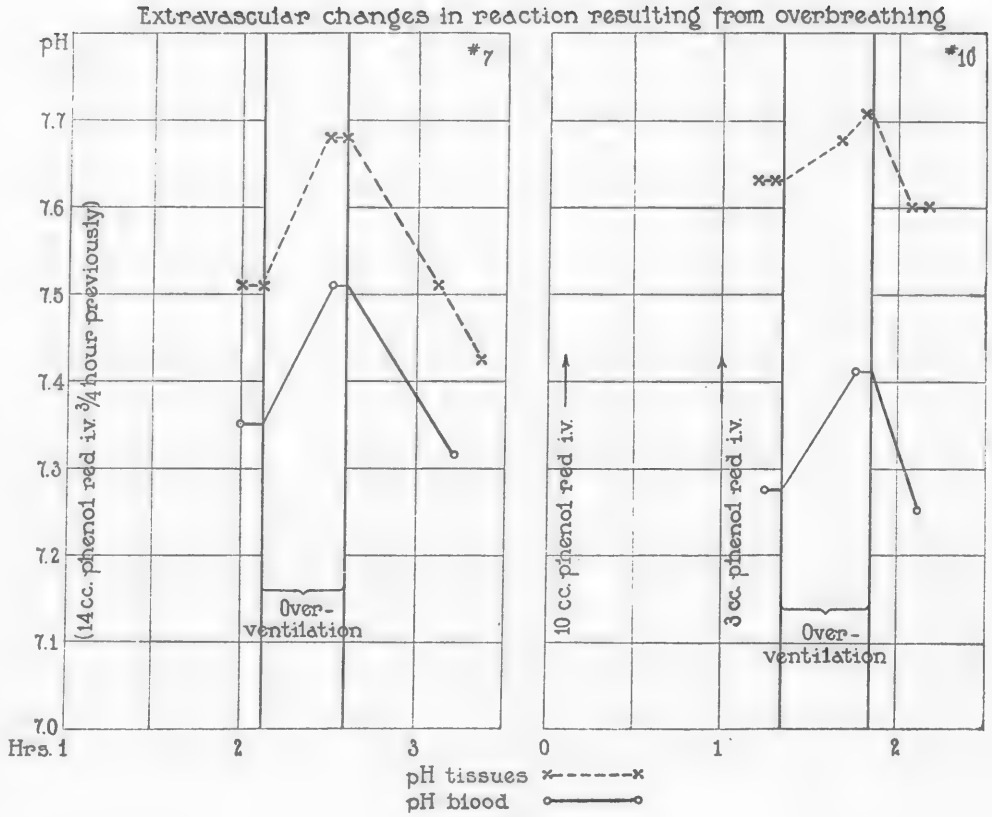

Extravascular changes in reaction resulting from overbreathing
pH
7.7
7.6
7.5
7.4
7.3
7.2
7.1
7.0
Hrs. 1
2
3
#7
(14 cc. phenol red i.v. ¾ hour previously)
Over-ventilation
#10
10 cc. phenol red i.v.
3 cc. phenol red i.v.
0
1
2
Over-ventilation
pH tissues
pH blood

CHART 4.

Artificial respirations begun at 32 per minute. 3.08—Animal decolorizing. *Surface hue* only slightly darker than *old rose.* 3.17—*Surface hue* now much darker and purpler than *eugenia red.* 3.31—Decolorization proceeds. *Surface hue* lighter than *eugenia red;* by wedge midway between pH 7.6 and 7.7. 3.32—*Second blood specimens taken.* 3.34—*Surface hue* by wedge between pH 7.6 and 7.7.

3.35—*Artificial respiration stopped.* Apnea of 50 seconds. 3.37—Respirations 146 per minute, *surface hue* much less purple. 3.59—Respirations have gradually become slower, now 54 per minute. *Surface hue old rose,* by wedge pH 7.5. 4.13—*Third blood specimens taken.* 4.20—*Surface hue alizarin pink,* by wedge pH 7.4+.

At 4.26 the animal was killed with ether and autopsied. It showed two localized pulmonary hemorrhages, 0.5 and 1.5 cm. in diameter respectively, one in each lower lobe.

Experiment 8.—Male rabbit No. 10 of 1600 gm. Given 1.3 cc. of urethane into tissues of back of neck at 12.30; 1 cc. more into peritoneal cavity at 2.00; and a few whiffs of ether during the tracheotomy, which was accomplished by 2.30. Between 2.21 and 2.25 10 cc. of phenol red was injected into an ear vein. The vital staining was even and deep, but owing to time lost in some accessory observations it was necessary to give 3 cc. more of the phthalein, at 3.30, to compensate for the decolorization that was taking place. 3.43—*Surface hue* slightly lighter than *eugenia red,* at pH 7.6 by wedge. 26 respirations per minute. 3.47—*First blood specimens taken.* 3.48—*Surface hue* again, pH 7.6.

3.50—*Artificial respirations begun* at rate of 28 per minute by alternating pressure and suction. 3.58—The blood in the superficial veins appears much purpler, so that these are more clearly seen. (The like observation was made in other experiments not reported here in detail.) 4.11—*Surface hue* by wedge between pH 7.6 and pH 7.7. 4.16—*Second blood specimens taken.* 4.19—*Surface hue* purpler and lighter than *eugenia red,* by wedge pH 7.7−.

Artificial respirations stopped. Apnea for only 35 seconds. Thereafter the respirations were at first rapid, 140 per minute, and then gradually slowed, to 82 per minute. 4.24—*Surface hue* lighter and only faintly purpler than *eugenia red.* 4.34—*Surface hue* slightly lighter and faintly purpler than *old rose;* by wedge method pH 7.6−. 4.38—*Third blood specimens taken.* 4.40—*Surface hue* by wedge pH 7.6−. Respirations 86 per minute. Observations discontinued. The rabbit was chloroformed for examination, on the same day. No lesions were found other than a liver coccidiosis.

Changes in the color of the connective tissue and cartilage could not at any time be made out.

While the alterations in reaction produced in these experiments (Chart 4) were not great they were sufficient for the purpose in hand, namely to determine whether an extravascular alkalosis is linked with

the blood alkalosis induced by overbreathing. Such is the case, the alterations in the blood reaction being closely paralleled by similar extravascular ones, as attested by the surface hue. In one of the two experiments the purple hue of skin flaps compressed between slides and viewed by transmitted light indicated the development of a more alkaline reaction of the connective tissue. No such change was to be seen in the ear cartilage.

DISCUSSION.

The experiments show that under the circumstances of acidosis due to the inhalation of carbon dioxide, and of alkalosis consequent on a blowing off of the gas, changes occur in the extravascular reaction which closely parallel those in the blood. Without doubt the extravascular changes largely involve the interstitial fluids, but during the acidosis concomitant alterations take place as well in the connective tissue and cartilage, the matrix tissues so readily rendered acid by the intravenous injection of acid solutions. During the alkalosis we noted connective tissue changes only, but this is not surprising since it was slight in degree and of brief duration. In our experience alterations in the reaction of cartilage follow rather tardily upon those of the connective tissue (*vide* Experiment 3); and this is especially true of the cartilage in the ear of the rabbit, which is almost avascular in the regions best suited to inspection. No observations were made upon the deep-lying organs; but one would expect from the results in rats given hydrochloric acid (6) that under the circumstances of acidosis due to carbonic acid the tendons would also become acid. Whether the reaction would also change in the liver, pancreas, and lymph nodes, parenchymal organs unaffected apparently by even the most extreme hydrochloric acid acidosis, is an interesting question. Quite possibly this would happen. For carbon dioxide manifests abilities to penetrate living tissue far beyond those of other acids (7).

An extravascular acidosis began to develop practically at once on the inhalation of the carbon dioxide, whereas it did not appear until a large quantity of hydrochloric acid had been run into the blood. This was to have been expected since the introduction of hydrochloric acid is compensated for in large measure by the elimination of carbon dioxide through the lungs, a possibility excluded when the gas is

being taken into the body by the pulmonary route. But over and above this difference one may attribute the prompt development of the extravascular CO_2 acidosis to the known ability of the gas to penetrate tissues rapidly (7). Evidence of such penetration was clearly to be seen in the course of our experiments. Where the circulation was unusually good, as for example about the almost imperceptible abrasions due to shaving (Experiment 5), the acidosis developed soonest; and where the vascularization was scanty, as along the line between ensiform and symphysis (Experiments 4 and 5) there it was seen last. The patching with color sometimes witnessed during the course of the inhalations (Experiments 3 and 5), and resembling that of outlying acidosis, was in reality consequent on a diametrically opposite state of affairs, those parts becoming most rapidly acid, and in consequence standing out in orange-red against a red background, which were most accessible to the acidotic blood, not least so.

The continuance of a carbon dioxide acidosis would doubtless lead to some accumulation of acid metabolites here and there in the body; but during the brief period of our experiments little evidence of such an event was obtained. The reaction of the blood and tissues swiftly changed for the better when the inhalations were discontinued, in significant contrast to the persistence of acidosis when hydrochloric acid had been administered. True, the reaction did not quite return to the normal even under the best of conditions (Experiments 5 and 6), and a compensated acidosis may well have been present and have endured for some time. To determine the actual case was no part of our work which had for sole aim the immediate influence upon the tissue reaction of alterations in the CO_2 content of the blood.

The normal reaction of the blood, as obtained from the femoral vein in the present experiments, varied from about pH 7.25 to slightly less than pH 7.4, whereas that from an ear vein, as noted in our preceding work (8), ranged from just below pH 7.4 to nearly pH 7.5. This very considerable discrepancy between the reaction of the two sets of specimens read in precisely the same way and by the same observer was a consistent finding. The fact may be recalled in this connection that the blood had come from very different regions. Since our object was to follow the relative, not the actual, variations

in intravascular and extravascular reaction the problem thus raised will not be discussed.

Hawkins has shown that urethane anesthesia results in an alkalosis (9); yet there can be no doubt that under uncomplicated conditions it tends to prolong the acidosis resulting from the inhalation of carbon dioxide (Experiment 4, Chart 2), as also that induced by hydrochloric acid. There is evidence that this comes about through a change in the respiratory center (10).

SUMMARY.

Breathing an atmosphere that contains the normal amount of oxygen but a large excess of carbon dioxide results in a tissue acidosis as well as one of the blood. The extravascular changes in reaction take place with far greater speed than when acidosis is induced with hydrochloric acid, and they do not persist as in the case of this latter but swiftly disappear when the animal breathes ordinary air once again. The changes parallel closely in magnitude and time those occurring in the blood. The same matrix tissues are rendered acidotic as when hydrochloric acid is administered.

The blood alkalosis that results from a blowing off of carbon dioxide is accompanied by an extravascular alkalosis. Under the circumstances of our experiments the connective tissue became more alkaline than ordinary but no change was noted in the cartilage, a fact to be explained by the slight degree of the alkalosis and its brief duration.

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THE QUANTITY OF CHOLESTEROL IN THE BLOOD SERUM OF THE GUINEA PIG AS AN INHERITED CHARACTER; ITS RELATION TO NATURAL RESISTANCE TO TUBERCULOSIS, AND TO TUBERCULOSIS INFECTION.

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The literature offers certain suggestions that changes in the distribution of cholesterol may be a feature of the pathology of tuberculosis.

Caldwell (1) analyzing tuberculous bovine lymph gland and liver tissue found two to three times the cholesterol content normal for these tissues. The percentage increase varied with the degree of caseation. Jaffé and Levinson (10) observed a marked increase in optically active fat in the tubercles of rabbits fed cholesterol in oil solution. Thus it would seem that there may be an increase of cholesterol at tuberculous foci and that the content of this substance may be carried still higher by administration of the substance.

Bacmeister and Henes (4) found either normal or increased values for blood cholesterol in tuberculous patients who were doing well and in good condition, while low figures were obtained in the stage of cachexia. Chauffard, Laroche, and Grigaut (5) obtained similar results. Weltmann (6) found normal cholesterol figures in early tuberculosis, but low ones in advanced, the amount falling rapidly in acute cases. Henning (7) obtained practically normal plasma cholesterol values when using the non-saponification method of cholesterol determination, while with the saponification method he obtained results uniformly much lower. He considered that this indicated the presence, in the blood of tuberculous individuals, of some substance other than cholesterol which gives the cholesterol color reaction but which is sensitive to alkali. Sweany, Weathers, and McCluskey (8) failed to find such a marked difference when using these two methods although the saponification method gave slightly the lower blood cholesterol values. This difference was not more marked, however, than have been noted (9) on a comparison of the two methods in conditions other than tuberculosis. They found that blood cholesterol values were highest in tuberculous patients who were doing well and low in patients doing poorly and losing ground.

Jaffé and Levinson (10), using rabbits infected with bovine type tubercle bacilli, found that following infection the blood cholesterol began to rise from a normal of 80 and 90 mg. per cent to 115 to 130 mg. per cent. In one animal this high value was maintained, while in the other animal it dropped before death to almost the original value.

The authors cited agree that the blood cholesterol is decreased in the more advanced stages of tuberculosis. In the earlier stages normal or increased values have usually been found. This refers to human beings and to rabbits. I have found no reference to determinations of blood cholesterol on either the normal or tuberculous guinea pig.

The inbred guinea pigs as used by Wright and Lewis (2), and Lewis and Loomis (3), are being maintained. In view of the variations in natural resistance to tuberculosis existing among inbred families of guinea pigs, it seemed not impossible that these animals might show some differences referable to their cholesterol metabolism. Dr. Lewis has kindly put his stock at my disposal for determinations of the cholesterol of the blood serum.

These experiments were therefore planned to determine the influence, if any, of an infection with the tubercle bacillus of bovine type on the cholesterol content of the blood of the guinea pig; and also to determine the normal cholesterol content of the blood of the available inbred guinea pigs, with particular reference to any familial variation.

EXPERIMENTAL.

It was found that there was a large individual variation in blood cholesterol, especially pronounced with the females. Observations were therefore confined to large groups of males.

Sixteen male guinea pigs from inbred Family 35 and the same number from inbred Family 13 were bled 1 cc. from the heart, and the 16 cc. of blood thus obtained was pooled and allowed to clot in the refrigerator. The clot was later broken up and the specimens centrifuged to obtain the serum. 3 cc. quantities were used in the determinations, and Bloor's method (9) was employed throughout with the modification that the amounts of alcohol-ether extractive were doubled because of the low serum cholesterol values in guinea pig blood. By using 20 cc. of alcohol-ether extract instead of 10 cc., colors readable in the colorimeter could be obtained. The cholesterol value obtained in this way had of course to be

TABLE I.

Uninfected Animals

Family No.	Serum cholesterol	
	Separate readings in mg. per cent	Average of readings in mg. per cent
Bled Feb. 4, 1926. Diet: Hay, oats, and mangels		
13	59.5 58.6 55.8	58
35	64.6 64.5 64.5	65
Feb. 5, 1926, diet changed to hay and soaked oats daily, cabbage once a week Bled Feb. 9, 1926		
13	56 57 57 55	56
35	69 66 71 69	69
Bled Feb. 15, 1926		
13	61 60 60 59	60
35	67 68 67	68
Feb. 18, 1926, infected with 1/10 mg. of a culture of bovine tubercle bacilli (Bov. 14), intraperitoneally		

TABLE I—*Continued.*

Infected Animals

Family No.	Serum cholesterol	
	Separate readings in mg. per cent	Average of readings in mg. per cent
Bled Feb. 23, 1926		
13	58 58	58
35	63 66	64.5
Bled Mar. 1, 1926		
13	57 56 57 57	57
35	75 73 68 68	71
Bled Mar. 8, 1926		
13	60 59 60 58	59
35	65 64 67 68	66
Bled Mar. 18, 1926		
13	59 59 60 59	59
35	67 67 68 68.5	68

TABLE I—*Concluded.*

Average for Total Experiment

	Family 35	Family 13
Before infection.....	67 mg. per cent	58 mg. per cent
After "	67 " " "	58 " " "

divided by two. Each specimen of serum was run in duplicate and the extractives in turn, were run in duplicate in most cases.

The animals were bled three times at intervals of 5 or 6 days. They were then inoculated intraperitoneally with a virulent culture of the tubercle bacillus of bovine type and again bled at intervals of 7 to 10 days after the infection had been established. Animals killed accidentally while bleeding were replaced, until the time of inoculation.

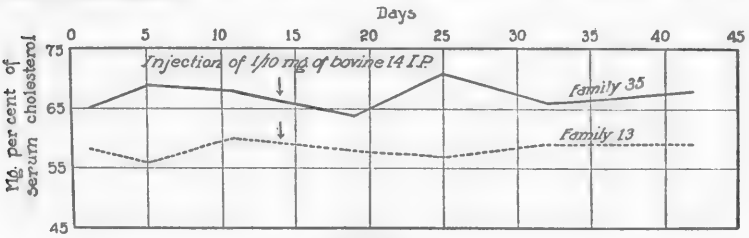

CHART 1.

The results of the serum cholesterol determinations are given in Table I, and graphically in Chart 1.

From these results it is evident that a tuberculous infection in guinea pigs produces no marked change in the serum cholesterol values. There was no rise at the onset of infection and no terminal fall was noted, although at the time of the last bleeding the animals were losing weight rapidly and had begun dying of tuberculosis. The animals in Family 35 showed uniformly higher serum cholesterol values than did the animals in Family 13. For reasons apart from present considerations, the diet was changed after the first bleeding of the period prior to infection and the diet then established was maintained throughout. The change was evidently without effect on the cholesterol content of the serum.

A further series of examinations was now conducted involving guinea

pigs from each of the four inbred families whose reactions to tuberculous infection are known. In this case a change of diet was also introduced following the first bleeding and 2 weeks before the second.

TABLE II.

Family No.	Serum cholesterol	
	Separate readings in mg. per cent	Average of readings in mg. per cent
Bled Mar. 30, 1926. Diet: Timothy hay, oats, and mangels		
13	57 57	57
35	66 66	66
32	64 61	62.5
2	49 51	50
Apr. 1, 1926, diet changed to alfalfa hay and oats, cabbage once a week Bled Apr. 13, 1926		
13	55 55	55
35	61 69	65
32	66 66	66
2	52 53	52.5

Since the previous results had made it apparent that tuberculous infection produced no changes in the serum cholesterol values in guinea pigs, the determinations this time were only on normal, uninfected animals. Eight male guinea pigs of about the same weight and age from each family were used for this experiment and the blood was handled in the same way as before. The results are given in Table II.

In this series, guinea pigs of Family 35 again showed a uniformly higher serum cholesterol content than those from Family 13 and the values obtained for each family are comparable to those obtained in the first experiment. Guinea pigs of Family 32 have values very close to those for Family 35, while the figures obtained for Family 2 are lower than those found for Family 13. On the basis of serum cholesterol the order of the families from high to low is 35, 32, 13, 2.

The substitution of alfalfa for timothy hay in the diet had no apparent effect on serum cholesterol concentration.

TABLE III.

No. of guinea pig	Family	Serum cholesterol	
		Separate readings in mg. per cent	Average for families in mg. per cent
1	13	50	50
2		48	
3		50	
4		52	
5	35	63	59.6
6		58	
7		59.5	
8		58	

In order to determine how wide might be the individual variations among the animals of Families 13 and 35, serum cholesterol determinations were run on four male guinea pigs from each of the two families. 8 cc. of blood was drawn from the heart of each animal and 3 cc. of serum was used in the determination, following the same method as in the previous analyses. The results are recorded in Table III.

In this series of determinations the serum cholesterol values ran uniformly lower than in the preceding series. This may have been due to the fact that early in May, 2 weeks prior to the bleedings, the animals were put on a succulent diet consisting of freshly cut clover and alfalfa. Practically the same differences prevail between the two families although on a lower level. In the first experiment the average values obtained were, for Family 35, 67 mg. per cent, and for

Family 13, 58 mg. per cent, a difference of 9 mg. per cent. In this series the difference noted in the average of the values is 9.6 mg. per cent. It is also to be noted that the individual variation appears to be from 4 to 5 mg. per cent. Comparison with the tables of the earlier experiments shows that this is within the limits of variation shown by the separate readings on the same blood sample and indicates that the individual differences cannot, with the technic employed, be regarded as significant.

DISCUSSION.

The results obtained have interest in several directions. It is clear that the progress of tuberculosis in the guinea pig is not marked by changes in serum cholesterol. This is in suggestive contrast to the observations on the human disease and to the experiments of Jaffé and Levinson on the rabbit. The differences may depend on a different relationship due to species, but it is also possible that the changes found by previous observers, which have been changes in the cholesterol content of the whole blood in most cases, may be due to changes in the corpuscles.

Any changes in diet under controlled conditions, as in Experiments 1 and 2, were without appreciable influence on the serum cholesterol. This was to have been expected since the cholesterol and phytosterol concentrations of the interchanged foods were probably not very different and the change being from one dry food to another the water exchange was probably not affected. The lower values obtained in the third series of observations might well be due to the succulent diet being fed at the time, or to other seasonal changes.

The familial differences in serum cholesterol have been consistent and are of wide interest. The families of guinea pigs used had been strictly inbred for many generations and may be regarded as homozygous. Differences between them in other respects are pronounced and constant. They relate to such characters as color, conformation, size, and fertility, and have been fully discussed by Wright (11). The concentration of serum cholesterol appears to be a well defined character peculiar to the family, and hence, considering the nature of the animal material, one controlled by the inheritance. We are familiar

with only one other recorded instance of an inheritable character related to a particular feature of the general metabolism. Benedict (12) has described peculiarities in the purine metabolism of the Dalmatian coach hound which have a similar relationship. It is of importance that our knowledge of similar instances should be multiplied as preliminary to a more definite association of genetics with physiology and pathology.

The families of guinea pigs employed for the present work possess different levels of natural resistance against infection with the tubercle bacillus (2). Wright and Lewis were led by a comparison of results with crosses between the families to assume that the total of differences found must be due to the interrelated operation of three or more separately inherited characters. The question now arises whether the serum cholesterol content is one of the three or more factors predicated by these authors. The order of familial resistance from high to low as found by Wright and Lewis was 35, 2, 32, 13. The order based on the serum cholesterol concentration from high to low is 35, 32, 13, 2. These relations make it plain that the cholesterol concentration cannot be the only factor in determining the resistance to tuberculosis, nor can it be the controlling factor.

SUMMARY.

1. Infection with tubercle bacilli of bovine type produced no alteration in the serum cholesterol content in guinea pigs.

2. Certain controlled variations in diet produced no changes in the serum cholesterol content in guinea pigs.

3. Inbred families of guinea pigs known to manifest differing resistances to tuberculosis gave differing serum cholesterol values, but no direct relationship between the two sets of phenomena could be established.

4. It seems probable that in the guinea pig the cholesterol content of the blood serum is influenced by inherited factors.

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STUDIES IN BLOOD COAGULATION.

I. Certain Characteristics of Coagulation and Their Measurement.

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Plate 1.

(Received for publication, August 23, 1926.)

In the study of a malignant transplanted tumor of the rabbit (1, 2) it has been observed that animals pursuing a rapidly fatal course of the disease frequently show as a terminal event evidences of an hemorrhagic state, with extravasation of blood into muscles, connective tissue spaces and serous cavities. Blood tests demonstrate prolonged clotting rate and imperfect clot retraction. In earlier stages, also, of this florid and of milder forms of the tumor are found changes in blood coagulability, less extensive and consistently related to various phases of the tumor growth. The findings are to be reported in Part II of this communication, it being necessary first to consider in detail the characteristics of blood coagulation that were investigated and the means adopted for their evaluation.

In estimating blood coagulability particular attention has to be paid to the circumstances of the technique employed, since they of themselves may influence the readings very considerably. This has been emphasized abundantly by others, referring to the markedly accelerating effect of "tissue juices" upon coagulation (3), the acceleration produced by various foreign substances, metal, glass, etc., coming in contact with the specimen during the test (4), the retardation in clotting rate resulting from periodic or continuous disturbance of the blood necessary in making observations (5), or from exposure to the air, also, the important part played by the temperature at which coagulation takes place (6). While the effects of extraneous factors of this sort may be maintained constant in a given technique and the readings obtained be of significance relative to each other, the

result may be to remove the scale of values so far from the basic rate of coagulation as to obliterate certain abnormalities that occur. For example, cases of hemophilia have been described (7–9) in which the clotting time of blood taken directly from the circulation was greatly extended, while specimens that had touched the tissues in being secured coagulated at normal rates. These were extreme instances but they illustrate the importance of obtaining as nearly as possible a basic clotting rate value; and for the present work where but slight changes in blood coagulability are frequently presented such factors have had to be eliminated from the tests, a condition not satisfied by by any of the procedures known to us. Effort has been made, accordingly, to develop methods with conditions of technique nearly neutral in all regards to coagulation. Indicators were sought for to serve as bases of measurement, phenomena occurring distinctively and spontaneously in blood coagulating at rest, rather than the arbitrary type of standard usually employed, such as certain degrees of firmness, adhesiveness, ductility or plasticity of the clot.

The problem at hand required consideration of two phases of the blood coagulative process, *i.e.*, clot formation and clot retraction. These will be dealt with separately; first, the characteristics of each will be discussed, and then methods of measurement described.

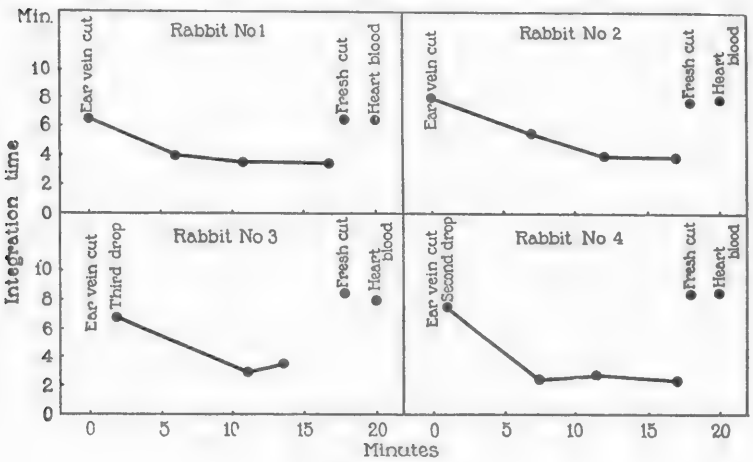
I. Clot Formation.

Experimental Basis.—A drop of freshly shed blood deposited under clear, bland oil and resting upon paraffin remains intact as a slightly flattened sphere and presents an exquisitely smooth and sensitive surface (Fig. 1, No. 1). Observed at room temperature, the sphere of blood remains without change of appearance for about 15 minutes, when, almost imperceptibly at first, retraction of clot begins as a minute dimple or wrinkle at one point or another (Fig. 1, No. 2), or as a warping of the whole specimen. Soon the distortions multiply and extend, and in the space of a minute or so the drop loses completely its symmetry and finish (Fig. 1, No. 3). Further retraction follows and bubbles of serum are extruded (Fig. 1, Nos. 4 and 5).

It is here evident that clot retraction commences in a specimen of blood only after a considerable period has elapsed since shedding, during which time the clot is being formed. Apparently, the fibrin

coagulum must undergo a certain amount of development or maturation before it becomes capable of retraction, and, once this condition has been attained, retraction proceeds rapidly. The duration of this period will be referred to as clot formation time, and, with the above preparation, it will be measured with the onset of retraction as end-point.

In the rabbit it is not practicable for repeated tests to take specimens of blood directly from the circulation by puncture of large vessels, and section of the ear vein must be employed.

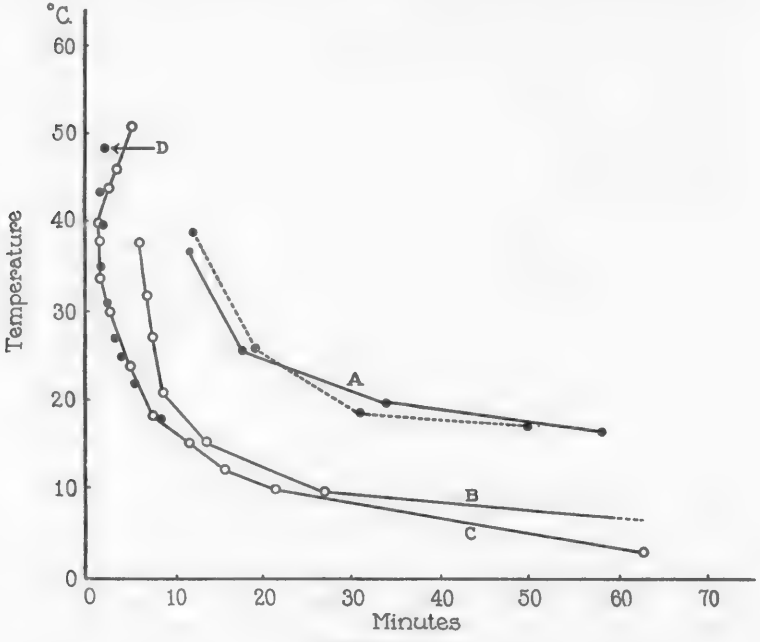


TEXT-FIG. 1.

Technical factors in this procedure which may influence the results are indicated by the following experiment: In each of four rabbits a series of drops from the same wound were tested for clot formation time; also, blood was tested from a fresh cut and from heart puncture. The results are charted in Text-fig. 1. It will be seen that clot formation quickens progressively in blood as it flows from the vein incision, up to a certain limit which may be twice as rapid as that at first. Blood from a fresh cut repeats the first reading obtained, as well as that drawn from the circulation without tissue contact. Thus, under circumstances prohibiting venepuncture, venesection may be used provided the first specimen of blood is taken. Skin puncture alone should not be used, as here the blood flows less rapidly and may be altered from the onset by admixture with "tissue juices."

The temperature at which the specimen coagulates requires control. The effects of temperature change upon the rate of clot formation are shown in Text-

fig. 2 at *A*. Curves from two rabbits are given, in each of which four specimens, taken simultaneously by heart puncture, were examined at different temperatures, ranging from 17–39°C. Clot formation is seen to vary in rate with the temperature, being greatly delayed at the lower levels. The relation, however, is not strictly direct, for the change in rate per degree change in temperature becomes greater as the scale is descended. Body heat (37.5°C.) is the logical condition for the test but this is too high to be maintained readily by the simple means of heat regulation to be described, and 32°C. has been selected. This temperature

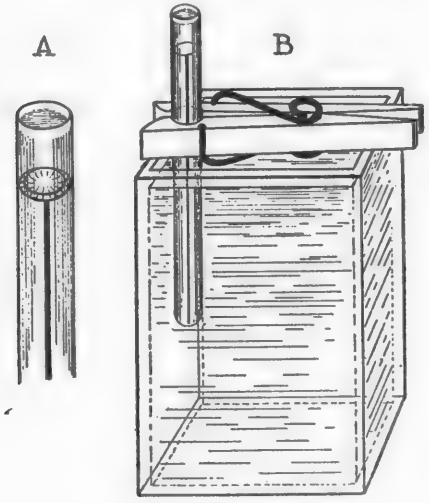


TEXT-FIG. 2.

is sufficiently high that the readings differ but slightly from those at 37.5°C. and, also, are affected inconsiderably by variations in temperature to the extent of 1° or 2° as may occur inadvertently in the technique.

Apparatus.—The articles required are a specimen platform, test-tube and holder, water bath and thermometer (Text-fig. 3). The platform is a thin, concave disc of metal, 1.0 cm. in diameter, supported upon a rod 10 cm. in length. The test-tube is so proportioned as to receive the platform disc readily, is 12 cm. in length and made of clear glass. The test-tube holder is of the type illustrated. A vertical sided jar of about 2 liters capacity serves as water bath.

The parts are assembled for use as follows: The platform disc is coated to at least 1 mm. thickness by dipping in melted paraffin several times, care being taken to preserve the concavity of the upper surface. The tube is filled with mineral oil (Squibb's) to within a few mm. of the top, and into it is dropped the platform, the disc of which will then lie about 1.5 cm. under the oil (Text-fig. 3, *A*). The tube, grasped in the holder, is now suspended part way in the water bath (at about 45°C.) and 5 minutes time is allowed for the column of oil to reach a stationary temperature (Text-fig. 3, *B*). The temperature of the layer of oil overlying the platform is then taken, the thermometer bulb first being warmed in the hand. It is desired to obtain in this section of the oil column a heat level of 32–33°C., and the tube



TEXT-FIG. 3.

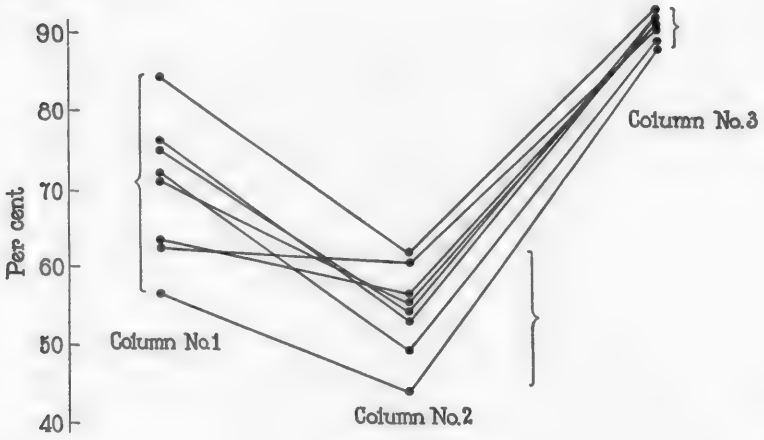
is further lowered or raised in the bath until the temperature is secured. Once adjusted, it will not fall appreciably during the test.

In dealing with a series of animals a rack of test-tubes and a larger water bath have been used. The thermometer is fixed in one tube with its bulb at the level of the platform discs, and by adjusting the height of the rack in the water, and lowering it slightly from time to time as the water cools, all tubes may be kept at the desired temperature for as long as 3 hours while they are being used.

Method.—A small quantity of blood is drawn with a syringe, and, with the point of the needle immediately under the oil, a single drop about 0.6 cm. in diameter is injected and allowed to rest upon the platform, care being exercised that it does not touch the sides of the tube. The size of the drop is unimportant except as the

larger specimens are more easily read. It is observed closely for the first evidence of clot retraction. Fine indentations in the surface indicating this are best seen by bringing the high light to play on the sides of the drop; and warping is detected from above. The interval of time elapsing between securing the blood and the onset of retraction is recorded.

The blood specimen is obtained in the rabbit usually from the ear vein. The skin surface here is shaved, cleansed and dried, then vessel dilatation is produced by applying warmth underneath (small electric light bulb) and a razor cut is made directly into the vein where it lies beneath the skin. Blood will spurt out, and the first that appears is drawn into a syringe or rubber bulb pipette (medicine dropper) for testing.



TEXT-FIG. 4.

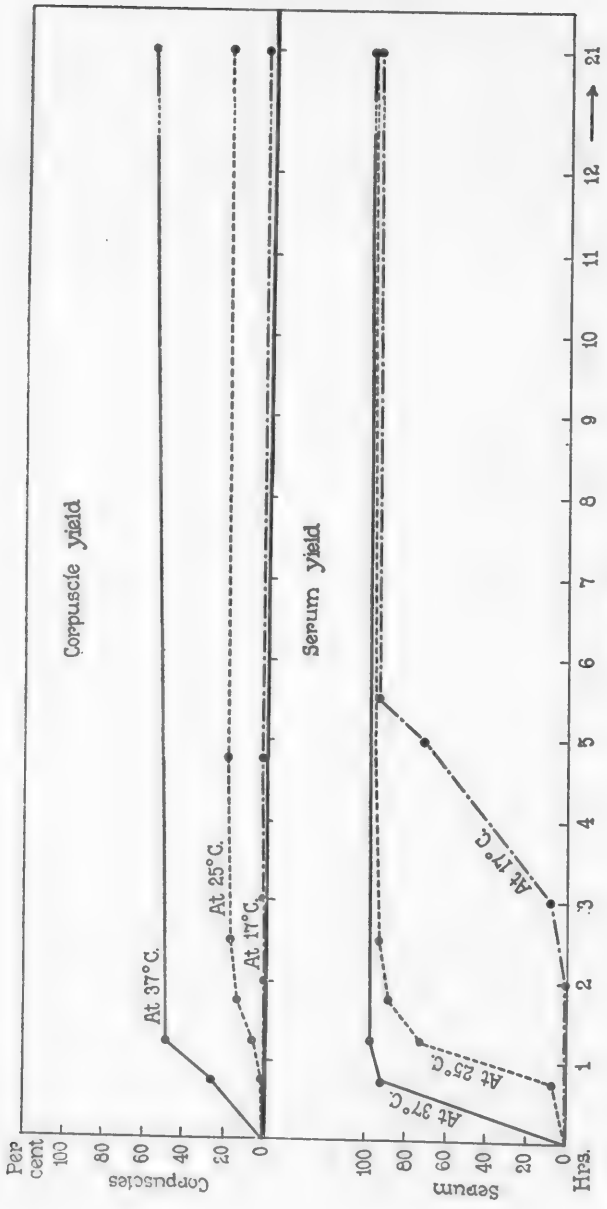
Results.—70, or one-third, of the number of rabbits whose clot formation time at 32°C. has been examined, were normal animals and gave readings between 4 and 8 minutes, averaging 5½ minutes. In those with various diseases the readings varied between 2 and 36 minutes. Incoagulable blood renders no end-point; the drop remains symmetrical and the corpuscles therein sediment, leaving a supernatant fluid of ground glass opacity.

Corpuscle sedimentation occurs also to a degree in certain blood specimens which go on to coagulation. This is accompanied by a prolonged clot formation time, although the latter is frequently found independently. The significance of sedimentation will be discussed below.

II. Clot Retraction.

Experimental Basis.—Extent of Retraction.—Since blood clot retracts with a loss of substance (serum and an amount of corpuscles), it is possible to study the characteristics of clot retraction quantitatively in terms of this substance. For example, if a known amount of rabbit blood be collected and allowed to coagulate completely, it will be found that the material which exudes from the clot amounts to about 70 per cent of the specimen; or, in a series of normal rabbits, between 55 and 85 per cent (Text-fig. 4, Column 1). If these exudates are then centrifugalized, to separate the corpuscles, and the amounts of serum compared, a lesser range of variation will be found (Text-fig. 4, Column 2). But it is logical to suppose that a given specimen of blood can deliver serum only in amount depending upon the available fluid (plasma) content. If, therefore, estimations of the plasma-corpuscle ratios in the blood of these animals be obtained (hematocrit test) and each of the above serum values be expressed in percentage of the plasma content, the range of results will be reduced to within narrow limits (Text-fig. 4, Column 3). It is evident, accordingly, that the normal extent of retraction of blood clot, expressed in terms of the relative amount of fluid lost in the process, is a fairly constant function.

Rate of Retraction.—The rate with which clot retracts, moreover, can be indicated by taking readings of this sort at intervals during retraction. Thus, in Text-fig. 5, the lower chart presents the curves of serum output of three specimens of blood from a rabbit. In order to vary the retraction rate each was placed at a different temperature to coagulate. Marked differences in rate are to be seen, the highest occurring in the specimen at the highest temperature; but the final readings, that is, the ultimate extents of retraction, in the three were the same. For comparison with these curves, the quantities of corpuscles which accompanied these serum yields at each reading during retraction, expressed in the same manner, are given in the upper chart of Text-fig. 5. Here again the curves demonstrate differences in rate, but the final values are not at all similar and vary also with the rate. It would appear that the total amount of corpuscles extruded from blood clot in retracting depends in some manner upon the rate of that process and in no wise upon its ultimate extent.


Corpuscle yield
Serum yield
At 37°C.
At 25°C.
At 17°C.
Per cent
Corpuscles
Serum
Hrs.
0
20
40
60
80
100
1
2
3
4
5
6
7
8
9
10
11
12
21

TEXT-FIG. 5.

Further indication that the total corpuscle output from blood clot is an index of clot retraction rate is to be had in the following experiments, in which clot retraction rate is altered artificially by various means.

Table I contains the data of experiments utilizing the accelerating influence upon coagulation of contact of foreign substances (glass and copper) with the blood. The corpuscle yield was read upon specimens from three rabbits, the blood being contained during coagulation in paraffin-lined tubes and covered with

TABLE I.

The Accelerating Effect of Foreign Body Contact on Retraction Rate.

	Corpuscle readings		
	Rabbits		
	A	B	C
	per cent	*per cent*	*per cent*
Specimen 1 (tube paraffin-coated and with oil)	6	15	47
Specimen 2 (tube omitting paraffin coat)	17	27	
Specimen 3 (tube paraffin-coated and with oil, added piece of bare copper wire)			74

TABLE II.

The Retarding Effect of Neutral Foreign Bodies on Retraction Rate.

	Corpuscle readings
	per cent
Specimen 1 (tube paraffin-coated and with oil)	47
Specimen 2 (the same as above; added one piece paraffin-coated copper wire)	28
Specimen 3 (the same as above; added three pieces paraffin-coated copper wire)	23

oil. With these as controls, readings of other specimens were made treated in all respects similarly, except that in two instances the paraffin lining was omitted, and in the third a fragment of copper was placed in the blood. The corpuscle yields in all of the latter were distinctly greater than in the controls.

The experiment in Table II made use of the retarding effect upon clot retraction of mechanical obstruction to the process. In comparison with a control reading, obtained as described in the above experiment, two other specimens from the same animal were tested in which there had been placed strands of copper

wire rendered neutral to coagulation by a coating of paraffin. The corpuscle reading from each of the latter was less than from the control and diminished in proportion to the number of wire strands present.

In Table III data are given concerning the retardation of clot retraction by exposure to air. Control readings were made as before in three rabbits and, for comparison, specimens were read from which the oil covering had been omitted. The corpuscle output was thereby lowered.

Agitation of the blood retards coagulation. In comparison with the usual controls in two rabbits (Table IV), specimens were tested similar to the others in all

TABLE III.

The Retarding Effect of Exposure to Air on Retraction Rate.

	Corpuscle readings		
	Rabbits		
	A	B	C
	per cent	*per cent*	*per cent*
Specimen 1 (tube paraffin-coated and with oil)	65	59	24
Specimen 2 (tube omitting oil)	38	45	14

TABLE IV.

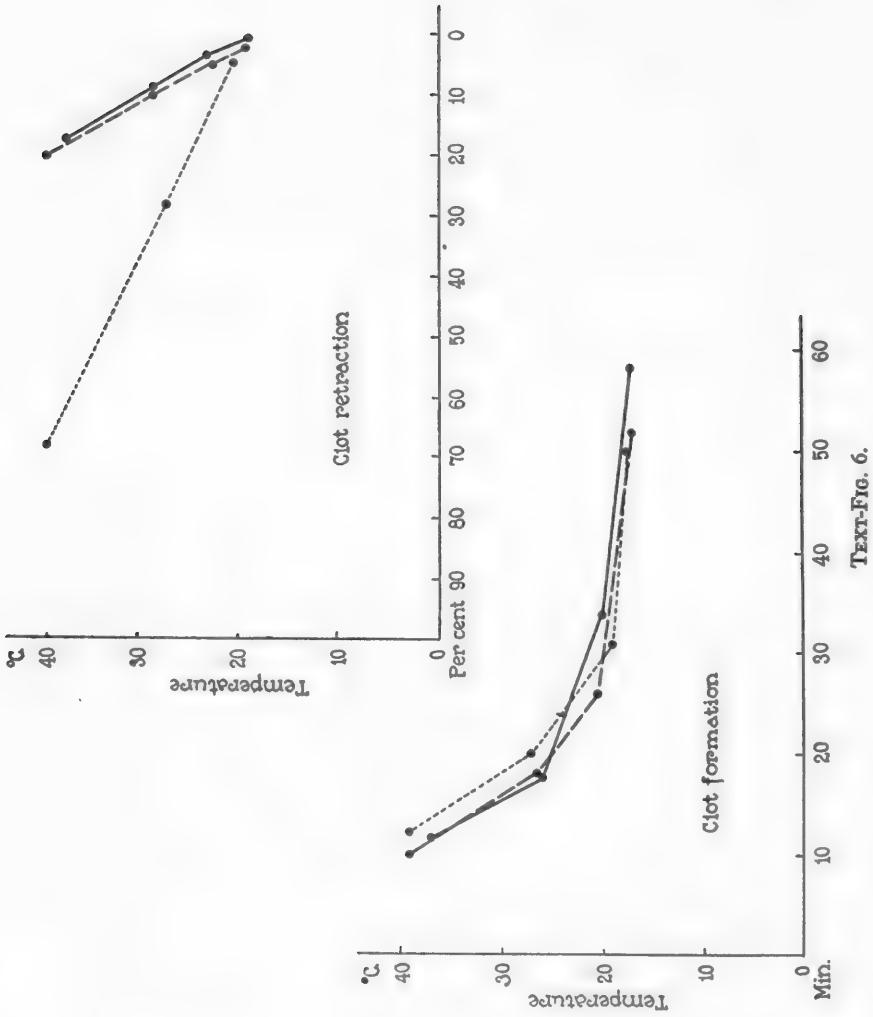
The Retarding Effect of Agitation on Retraction Rate.

	Corpuscle readings	
	Rabbits	
	A	B
	per cent	*per cent*
Specimen 1 (tube paraffin-lined and with oil)	47	31
Specimen 2 (the same as above)	49	
Specimen 3 (the same as above, stirred 1 min. after shedding)	29	23
Specimen 4 (the same as above, stirred 2 min. after shedding)		16

respects except that shortly after shedding the blood was slightly agitated. This had the effect in each instance of reducing the corpuscle yield.

Still further and surer proof of the significance of the corpuscle reading as an index of clot retraction rate is had from experience with the value in disease, for wherever retraction was found retarded as indicated by abnormally low intermediate serum readings, the total corpuscle yield was always depressed.

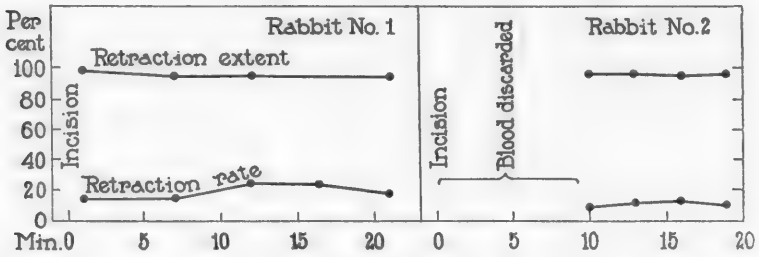
The means is presented, accordingly, of estimating the rate and the extent of blood clot retraction: The total fluid yield from clotted blood is separated into its serum-corpuscle fractions and, properly corrected, the corpuscle value indicates the rate and the serum value the extent of clot retraction.


Clot retraction
Per cent
0
10
20
30
40
50
60
70
80
90
Temperature
°C
10
20
30
40
Clot formation
Min.
0
10
20
30
40
50
60
Temperature
°C
10
20
30
40

TEXT-FIG. 6.

Relation of Clot Retraction to Clot Formation.—We may proceed to examine the behavior of clot retraction in its relationship to that of clot formation.

In Text-fig. 6, on the right, are curves of retraction rate secured from blood of three rabbits. In each instance four specimens were taken and allowed to coagulate at temperatures extending from 17–38°C. It will be seen that not only does rate vary with temperature but the effect is proportional, *i.e.*, each curve is virtually a straight line. In Text-fig. 6, on the left, are curves of clot formation rate from these animals secured under the same conditions of temperature (see also Text-fig. 2, *A*). Here the relation between rate and temperature is represented by a line of hyperbolic form. Moreover, while the formation rates at corresponding levels are nearly alike in the three animals, one of them gave a retraction rate widely separated from those of the other two. Apparently, clot retraction and clot formation behave quite independently in rate.

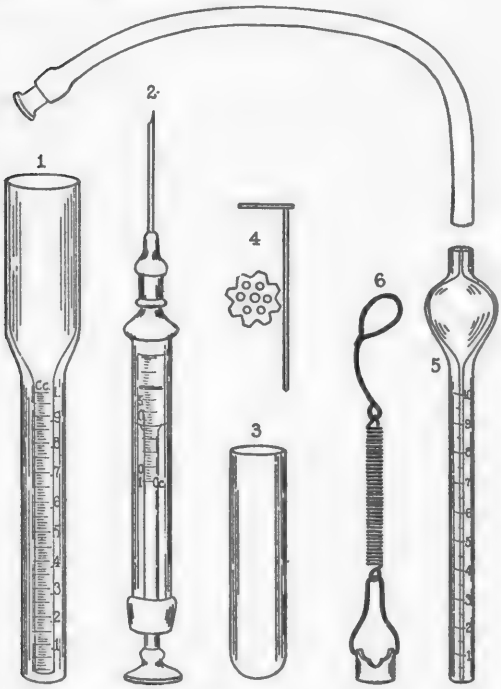


TEXT-FIG. 7.

This individuality of rate of the two phases of coagulation may be demonstrated in another way: Five specimens of blood from an animal were allowed to coagulate at different temperatures, thus, Nos. 1 and 2 at 17°C., No. 3 at 25°C. and Nos. 4 and 5 at 37°C. As coagulation proceeded, Specimens 2 and 4 were observed closely and at the completion of clot formation they were interchanged, *i.e.*, No. 2 was placed at 37°C. to undergo retraction, and No. 4 at 17°C. After coagulation was complete, the yields from all specimens were read (Fig. 2). The corpuscle yields from Nos. 1, 3 and 5 varied as would be expected with the temperature; the corpuscle yield of No. 2 was a duplicate of that of No. 5, and that of No. 4 was a duplicate of No. 1. Evidently, specimens which retracted at the same temperature retracted at similar rates, without reference to the temperature (rate) at which clot formation took place.

It has been demonstrated (Text-fig. 1) that clot formation is markedly accelerated by contact of the blood with the tissues. In Text-fig. 7 are given clot retraction readings in two rabbits, the specimens being taken successively from the same wounds. The curves are practically horizontal. From this it is evident that clot retraction is not affected as is clot formation by tissue kinase.

Apparatus.[1]—Record syringe (Text-fig. 8, No. 2), of 1.2 cc. capacity, graduated in 0.2 cc. divisions. Test-tube (Text-fig. 8, No. 3), of 1.0 cm. internal diameter and 5.0 cm. length. Graduated centrifuge tube (Text-fig. 8, No. 1), of 1.0 cc. capacity. Separating disc (Text-fig. 8, No. 4), which is a perforated metal disc of the same diameter as the test-tube. Hematocrit (Text-fig. 8, Nos. 5 and 6), described elsewhere (10, 11).



TEXT-FIG. 8.

TEXT-FIG. 9.

In preparation for the test the glassware is carefully cleaned, particular attention being paid the syringe that it contains no residue of blood and is dry. The test-tube is given a heavy lining of paraffin, thus: It is dipped into melted paraffin

[1] These instruments may be obtained from the Gottlieb Greiner Company, New York City, with the exception of the hematocrit which is manufactured by the Arthur H. Thomas Company, Philadelphia, and Firma Arno Haak, Jena, Germany.

which has been cooled almost to the point of thickening, and on being removed is immediately placed inverted under cold running water. The external surface is then wiped free of paraffin and the inner surface examined to make sure that it has been coated entirely and is free from water.

Method.—The syringe is filled to 1.2 cc. with blood (for the rabbit see technique of collecting blood above) and 1.0 cc. of this is deposited precisely in the bottom of the test-tube (thus is avoided error from the dead space air bubble in the syringe). A few drops of mineral oil are then added to cover the specimen and it is placed at 37°C. for coagulation.

An hematocrit test is now performed.

From 5 to 6 hours after collection of the specimen, the test-tube is removed from the incubator, covered with the separating disc and inverted into the centrifuge tube (Text-fig. 9). This is then centrifugalized for 30 seconds at the lowest speed (about 500 R.P.M.) necessary to displace the fluid contents of the test-tube completely through the separating disc into the graduated tube. Now, the test-tube, separating disc and blood clot are removed, and the graduated tube, containing the fluid yielded from the clot, is centrifugalized at high speed (about 3000 R.P.M.) for 5 minutes to separate corpuscles from serum.

The amounts of corpuscles and serum are read from the scale on the centrifuge tube (Fig. 2), and these values are corrected with reference to the hematocrit result, to give the final readings, as follows:

If x = final serum reading, representing retraction extent,
y = final corpuscle reading, representing retraction rate,
a = serum reading from tube; for example, 0.56 cc.,
b = corpuscle reading from tube; for example, 0.18 cc.,
and c = amount of corpuscles in whole blood; for example, where the hematocrit reading is 40 per cent, the corpuscle content of the specimen is 0.40 cc.,

then $x = \frac{a}{1.00 - c}$, for example $x = \frac{0.56}{1.00-0.40} = 0.93$ (read 93 per cent),

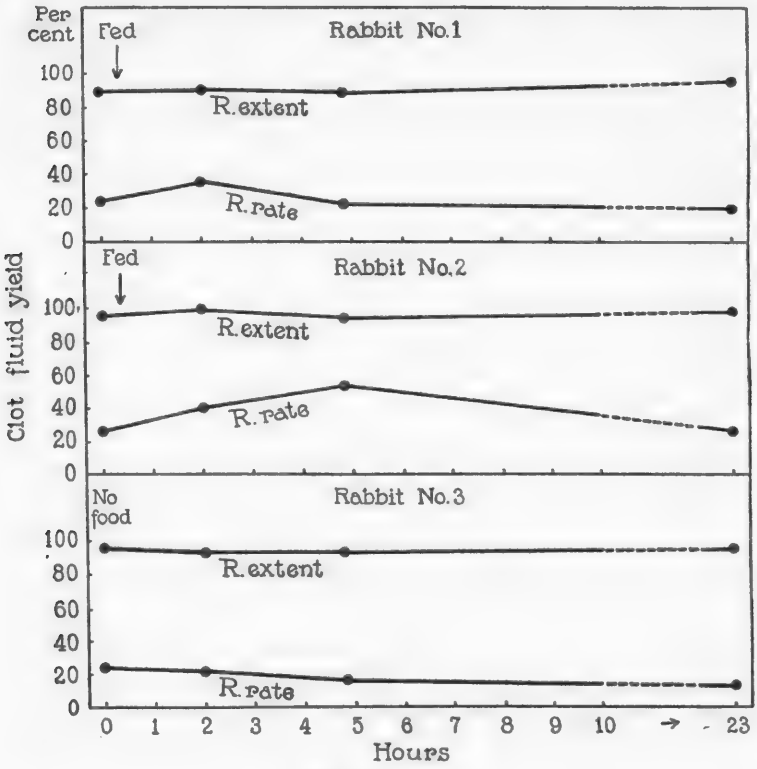
and $y = \frac{b}{c}$ for example $y = \frac{0.18}{0.40} = 0.45$ (read 45 per cent).

Certain technical points require emphasis:

It is of first importance in securing the specimen that no more than 20 seconds shall elapse between its leaving the circulation and entering the test-tube, for agitation of the blood in the early stages of coagulation produces marked reduction in coagulation rate and renders the reading misleading (Table IV). This small quantity of blood may be obtained rapidly enough when withdrawn by venepuncture, but in the rabbit where it is collected from the pool of blood issuing from an incision the task is often difficult. When there is any question about the rapidity of collection, a second specimen should be taken and, if the reading is higher, adopted. Once the blood is in the tube further haste is not imperative, although no more than 2 minutes should intervene before placing it in the incubator.

If hemolysis occurs and the serum is found tinted, no reading is to be made, since bacterial activity has probably altered the specimen. For this reason the incubation is limited to 6 hours.

Where it is obvious from inspection after incubation that clot retraction has occurred to a slight extent only, it is better to remove the accumulated serum by pipette for measurement rather than by centrifugalization, for such clots are soft



TEXT-FIG. 10.

and give up additional fluid readily on pressure. Where clot formation fails or takes place only in portions of the specimen, it is of course impossible to measure clot retraction and fluid readings become valueless. In these cases there will usually be found upon the separating disc either no clot at all or misshapen clot fragments; even where the clot appears normal the condition may be detected,

since the serum reading is abnormally low (under 87 per cent) and the corpuscle value high (over 30 per cent). Such a result is, of course, indicative of an exceedingly defective coagulation and is of value qualitatively.

At the time of examination the subject should be fasting. The apparent accelerating effect of food ingestion upon clot retraction is illustrated in Text-fig. 10.

Results.—Records are at hand of 810 readings of clot retractility. The normal rate of clot retraction lies between the values 40 and 60 per cent (corpuscle output) and the normal extent between 87 and 100 per cent (serum output). In disease, instances were found of all grades of depression of these values, to 0 per cent in non-retractile blood clot.

The margin of error from various technical sources allowed in the interpretation of readings is 10 per cent of the rate value and 2 per cent of the extent value.

DISCUSSION.

The factor here measured as clot formation rate is but another expression of the same function of the blood coagulative process as the clotting rate values of other methods. This is shown by their similarity of behavior in the presence of various disturbing influences, such as tissue kinase and foreign body contact with the blood, but more particularly by the type of their reaction to temperature change. Thus, the temperature curve of clot formation as given in Text-fig. 2, at *A*, runs parallel with those at *B*, *C* and *D*, which are constructed from data published respectively by Lee and White (7) with the tube inversion method of Howell, by Addis (6) with his modification of the hanging drop method of Brodie and Russell, and by Dale and Laidlaw (12) with their shot tube procedure. However, judging from the positions occupied by the curves relative to each other, the end-point of clot formation is situated further along in the process of coagulation and would logically have the advantage of magnifying changes occurring in coagulation rate. In this procedure, also, the conditions under which the specimen resides while coagulating are nearly neutral, and the rate obtained may be considered as practically basic for the blood examined.

Sedimentation of corpuscles in the drop of blood tested is a phenomenon especially to be noted. It has been observed in specimens of coagulating blood by others, and is the cause of production of the

"buffy coated" blood clot (13, 14). Addis (15) found it associated with prolonged clotting time in extremely ill, septic patients. Lee and White (7) noticed it in various diseases, and so frequently was it accompanied by delayed clotting time, that the phenomenon was used as confirmatory evidence of the latter condition. Sedimentation has been found here also followed by an extended end-point of clot formation, but it probably has particularly to do with the beginning-point and indicates delay in the onset of coagulation. This can be shown as follows: If a specimen of blood is chilled directly after collection, the corpuscles will soon commence to gravitate and this will continue until the temperature is elevated and coagulation is allowed to proceed; should, however, the chilling be postponed for 1 minute after shedding, to allow coagulation to begin, and is then instituted, sedimentation will not take place much as the clotting end-point may be delayed by the low temperature. Also, it has been found that, where corpuscle sedimentation occurs in disease, examination of the blood by means of the wire loop of Buckmaster (5) demonstrates marked delay in the appearance of certain alterations in the specimen peculiar to the onset of coagulation. In the gravitation of the corpuscles, accordingly, is presented a means of detecting retarded initiation of coagulation, a factor in addition to the estimation of clot formation rate.

Clot retraction is a function which depends upon physical principles quite separate and distinct from those underlying clot formation, since, as has been demonstrated, they behave differently in the presence of various disturbing influences.

It has been necessary to resort to indirect means for the estimation of clot retractility. Direct measurement of size alterations in the clot itself with any degree of accuracy is well nigh impossible, since the pattern of retraction is extremely variable, particularly in the early stages, and measurement in one or two diameters alone is not sufficient. However, the amount of fluid yielded from the clot during retraction is as logical a measure and more readily applied; either procedure must take into consideration the plasma-corpuscle ratio of the blood and the amount of corpuscles extruded with the serum, in order to narrow the range of normal values and distinguish the abnormal. The reason is not apparent for the parallelism found between the rate of clot retraction and the quantity of corpuscles which escapes from

the clot. This has probably something to do with the mechanics of fibrin retraction. In early tests, an additional serum reading was made 1 hour after collecting the blood and compared with the final serum reading as an indicator of clot retraction rate. But this was abandoned when the significance of the corpuscle output was appreciated, and, not only has the technique been simplified thereby, but the corpuscle reading has proven much more delicate in determining changes in rate.

SUMMARY.

The gross phenomena of normal blood coagulation have been studied for the purpose of obtaining methods for estimating certain characteristics of coagulation in connection with a malignant tumor of the rabbit. The technique is described for the estimation of clot formation rate and of clot retraction rate and extent. The range of normal and abnormal values secured by these procedures is given.

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FIG. 1.

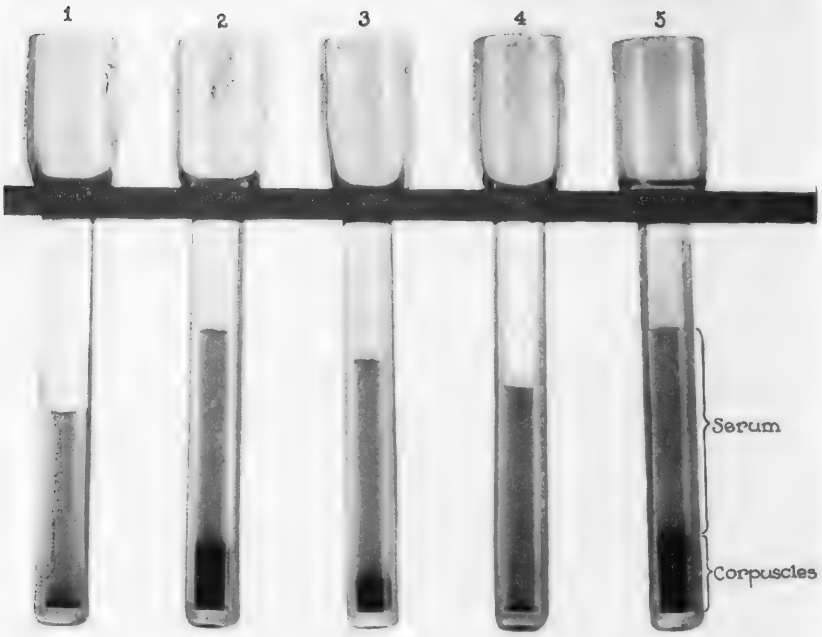

FIG. 2.

(Van Allen: Blood coagulation. I.)

STUDIES IN BLOOD COAGULATION.

II. Blood Coagulability in Malignant Tumor and Other Diseases of the Rabbit.

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Alterations in the coagulability of the blood will be described which occur in various stages of the growth and extension of a malignant tumor of the rabbit, and, as a basis of interpretation, there is explained also the behavior of coagulation in other diseases and abnormal states of this animal. The methods of test employed have been described (1), and include estimation of clot initiation and clot formation rate and the rate and extent of clot retraction.

I. Disease in General.

Relation of Blood Coagulability to Presence and Extent of Disease Lesions.

Experimental.—100 adult male rabbits, taken from stock as received from the breeders, were placed under observation for 1 or more weeks.[1] All grades of physical condition were presented, although the great majority were free from external evidences of disease. The blood coagulability of each animal was then tested, and autopsy examination was made within 24 hours afterward. A comparison of the readings with the anatomical findings follows.

Results.—As regards clot formation rate, the readings in almost every instance were within the limits of normal variation, *i.e.*, between 4 and 8 minutes. Five exceptions to this presented, and all of these were in extreme stages of disease: Two gave clot formation time of 10 minutes, and showed at autopsy fatty degeneration of the kidneys and profound cachexia, which changes were probably secondary to an occult disease process. One rabbit with extensive coccidiosis of the

[1] These rabbits are part of a larger group being used for other experiments in the study of normal and abnormal organ weight variations.

liver, another with snuffles complicated by septicemia and a third markedly emaciated but without discernible lesions gave delayed clot initiation and an irregular clot formation allowing of no distinct reading.

The extent of clot retraction was also normal in the majority of the animals, *i.e.*, reading between 87 and 100 per cent. In the last

TABLE I.

Autopsy finding		Analysis of groups on a basis of clot retraction rate				
		40–60	30–40	20–30	0–20	No reading obtainable
		per cent	*per cent*	*per cent*	*per cent*	*per cent*
Rabbits grouped on a basis of stress of disease	11, without lesions and in good physical condition	91	9			
	10, with healed lesions of various types	80	20			
	24, with coccidial lesions, small and of questionable activity	25	33	29	13	
	13, in process of recovery from various types of infections		32	60	8	
	18, with purulent infections, snuffles, mastoiditis, etc.		5½	11	78	5½
	8, with coccidial lesions, numerous and obviously active			40	50	10
	4, emaciated, with fatty degenerative lesions of kidneys			25	75	
	2, emaciated, with active parasitic lesions of kidneys				100	
	2, emaciated, with focal lesions of appendix				100	
	3, emaciated, but without discoverable disease process				67	33

three rabbits above mentioned occurred the only abnormal values, 85, 85½ and 81 per cent, respectively.

Clot retraction rate, on the other hand, was extremely variable, both in rabbits obviously ill and those without symptoms. The autopsy findings are correlated statistically in Table I with the retraction rate values. On the left, the rabbits are grouped according

to the type of disease found and the groups are arranged in order on a basis of degree of physical deterioration (disease stress) evident. On the right, analysis is made of each group from a standpoint of the clot retraction rates, and for this purpose four divisions are used. It will be seen that in the highest rate division, 40 to 60, fall 91 per cent of animals without lesions and in good physical condition, also, 80 per cent of those with healed lesions only and 25 per cent with insignificant coccidial lesions. As analysis proceeds thus from group to group dealing with disease of increasing severity, the distribution of rate values plainly shifts across the scale, and the last five groups lie entirely within the lowest rate division, 0 to 20. The animals which fall principally in the intermediate divisions, 20 to 30 to 40, are those with moderately active disease or in process of recovery from severe infections. Retraction rate could not be measured in three rabbits, those last referred to above, because of the fragmentary nature of the coagulative process.

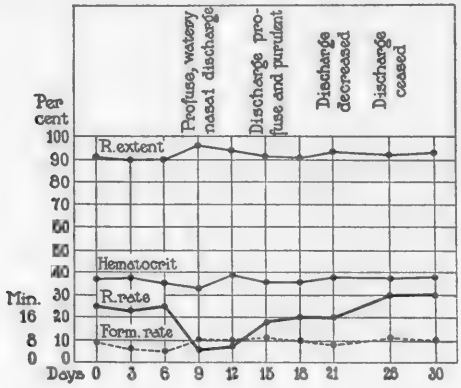
Five rabbits are excluded from the table for special mention: Two had been inoculated intratesticularly with the spirochete of yaws and had an early, active orchitis. At autopsy no other lesions were found. The retraction rates were normal, 42 and 48 per cent. Two rabbits had well developed goiter, were otherwise in good condition and gave readings, 45 and 56 per cent, within the normal range. One rabbit although a female was included in the series to illustrate a point of interest. She was abundantly fat and sleek and presented nothing pathological beyond a small, healing *cuniculi* papule on the genitalia. Nevertheless, the clot had an extremely low retraction rate, 9 per cent; at autopsy pregnancy at about the 10th day was discovered.

Relation of Blood Coagulability to Course of Disease.

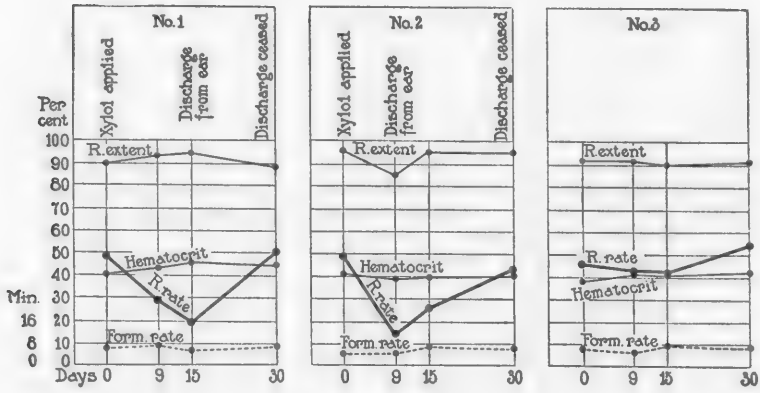
Experimental.—The behavior of blood coagulation was investigated during the course of various abnormal and diseased states, including instances of spontaneous infections, non-bacterial lesions and artificially induced affections of the blood coagulative mechanism. In each case, periodic tests were made and symptoms recorded, with autopsy examination at the termination of the experiment.

Findings.—Brief protocols of typical examples of each disease are given:

Snuffles.—Text-fig. 1 is the chart of a rabbit which had been followed for some time because of chronic snuffles. The infection persisted for about 2 months, gradually cleared, and the nasal discharge had ceased, when abruptly a relapse set in with profuse discharge and sneezing and the animal passed through a



TEXT-FIG. 1.

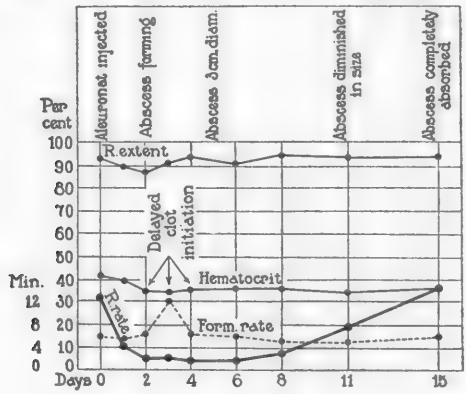


TEXT-FIG. 2.

second typical course of the disease, this time lasting 3 weeks. At autopsy, the nasal passages were free from inflammation; the right middle ear spaces contained a small quantity of inspissated pus. There was no other disease. Evidently the rabbit had had chronic otitis in addition to the attacks of snuffles. The period of snuffles remission only is included in the chart of blood findings. The rate of

retraction had been distinctly below normal and dropped still farther with the redevelopment of rhinitis. Later, as the infection cleared, the curve rose to its previous level. The readings of retraction extent and clot formation rate showed throughout no variations beyond the normal range.

Auditory Canal Abscess.—The charts are given in Text-fig. 2 of three rabbits from a group of fifteen which were being followed as controls for other purposes. At this time xylol was being used routinely painted on the inner surface of the ear to produce vessel dilatation for the collection of blood specimens, and in two instances, Rabbits 1 and 2, the xylol extended accidentally into the auditory meatus. The applications were discontinued immediately in favor of other methods, but the inflammation in the canal persisted and an abscess developed in its recesses. Rupture occurred in 2 weeks, with drainage of pus for another week. Rabbit 3 is given for comparison, for here no irritation arose, aside from a



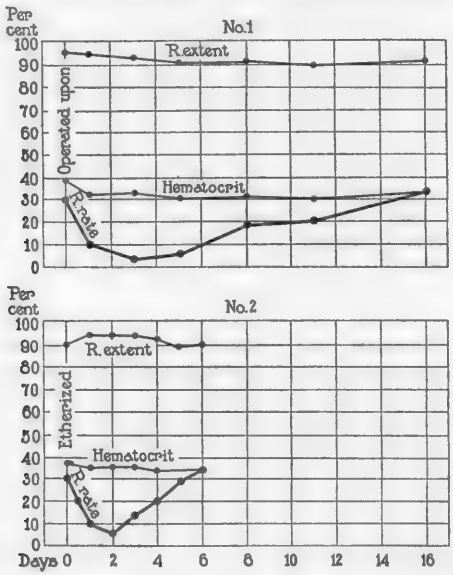
TEXT-FIG. 3.

slight erythema of the pinna. From the charts, it is to be seen that in the cases of the first two rabbits clot retraction rate fell after xylol application to a low level on the 15th and 9th days respectively, and with rupture of the abscess the reading began to rise and reached normal with the healing of the lesion. Retraction extent in the first rabbit was elevated in the early stage of inflammation, but in the second, where the rate drop had been more sudden and pronounced, the extent reading also fell temporarily. Both rate and extent of clot retraction in the third rabbit remained during this period within the normal limits. Clot formation showed no abnormalities in any of the animals.

Subcutaneous (Sterile) Abscess.—Six rabbits were injected either subcutaneously or intrapleurally with 0.5 cc. of a 10 per cent suspension of aleuronat. In each, pus accumulated at the site of injection, and in two of the animals which were allowed to live for 3 weeks the abscess was absorbed and no trace remained except

slight local increased vascularity. The record of one of the latter is represented in Text-fig. 3, the two being similar in all respects. The curve of retraction rate fell sharply within 24 hours after injection and remained at a low level for 6 days during the period of abscess development. Then the curve began to rise and by the 15th day was at normal, when the lesion was healed. The extent of retraction did not leave the normal range. Clot initiation and formation were delayed slightly at the height of the inflammatory reaction.

Operative Incision.—Four rabbits were operated upon under ether anesthesia with sterile precautions for the purposes of other work. In two the spleen was removed and in the others laparotomy only was done, as control. The wounds



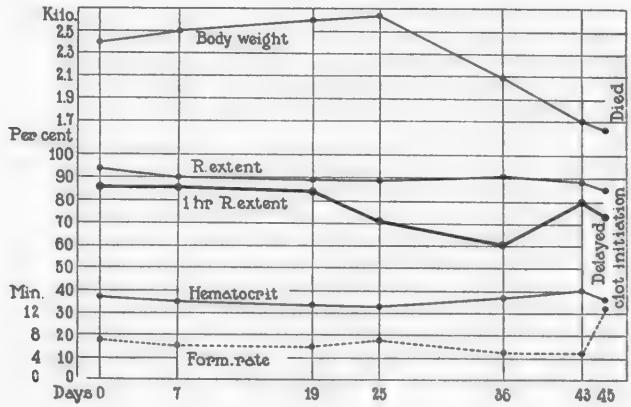
TEXT-FIG. 4.

healed *per primam*. The coagulation curves of one of the control rabbits, which were typical of all, are given in Text-fig. 4, No. 1. Immediately after the operation the rate of retraction dropped and reached a low point on the 3rd day, when it began to return and attained its original level on the 16th day. The extent of retraction remained stationary throughout. No clot formation readings were made.

Ether Anesthesia.—In order to ascertain the part played by ether in the preceding results, two animals were anesthetized for a period of ½ hour. The subsequent behavior of clot retraction in both was the same; the curves of one are given in

Text-fig. 4, No. 2. The rate became retarded in a manner similar to that in the chart above, but returned much more rapidly to normal.

Nephritis.—The chart is given in Text-fig. 5 of a normal rabbit which developed nephritis spontaneously while under observation. The animal had been in good health and gained steadily in weight until the 4th week of observation, when it began to grow thinner without other symptoms. 1000 gm. were lost before death 3 weeks later. At autopsy, the body tissues were found dehydrated and wasted; the only lesion presenting was with reference to the kidneys. These organs were markedly swollen, pitted and intensely yellow, with a fine surface stippling. On section, the cortices were found widened, of an opaque yellow color and without architectural markings. The medullæ were pale and presented radial grey streaks. From the chart, it will be seen that clot retraction rate



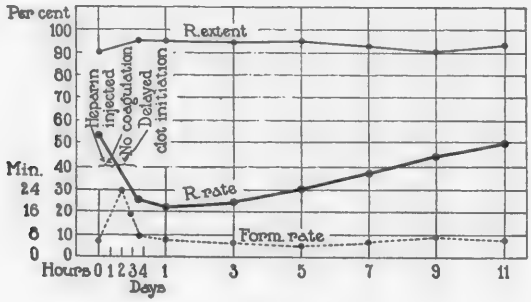
TEXT-FIG. 5.

(estimated by a 1 hour serum reading in comparison with the final serum output, a less sensitive index) showed marked retardation on the 25th and 36th days, a return toward normal on the 43rd day, and another drop before death. Retraction extent and formation rate showed no abnormalities until the final reading, when the latter was delayed.

Specific Affections of the Blood-Coagulative Mechanism.—Effort was made to reproduce experimentally conditions of blood coagulability imitating those in the hemorrhagic diatheses, hemophilia and thrombocytopenic purpura.

The properties of the blood in hemophilia are said to be obtained temporarily in animals by the injection of heparin (2), a phosphatide extracted from the liver by the process of Howell, that is, clotting time is prolonged but, when coagulation ensues, the clot retraction appears normal. In the present study seventeen rabbits

were used and heparin,[2] at a concentration of 0.5 per cent in physiological saline solution, was injected intravenously in amounts from 5 to 40 mg. per kilo body weight. Both single and series of doses extending over long periods were employed. In none of the animals appeared symptoms of any sort referable to the drug, nor did microscopic examination of the bone marrow, spleen, liver and other organs reveal changes. Marked alterations in blood coagulability were presented however, shown typically in Text-fig. 6. Here a single dose of 17 mg. per kilo was injected. The blood, examined immediately and again after 1 hour, failed to coagulate. 2 hours after treatment a specimen gave moderately delayed initiation of coagulation and formation time of 23 minutes. At 2 hours, 40 minutes, these two functions were still tardy but became normal 1 hour later. Although clot retraction was observed to occur in portions of specimens as early as 2 hours after injection, the process was so imperfect as to exclude estimation. At 3 hours, 40 minutes, the first measurement was obtainable and gave a considerably reduced



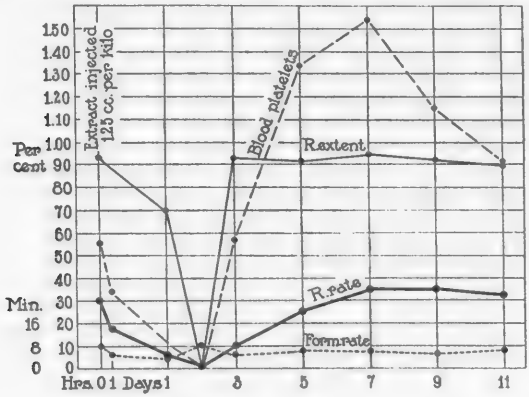
TEXT-FIG. 6.

rate and a normal extent. Further depression in rate was found after 24 hours, and then this function returned gradually to its original level by the 11th day. The other factors of coagulation were normal after the initial interference. These alterations in the blood varied in degree among the animals with the dose employed. After repeated treatments (20 in 60 days, of 40 mg. per kilo) rabbits appeared to have become somewhat resistant to the drug, although this was not consistent.

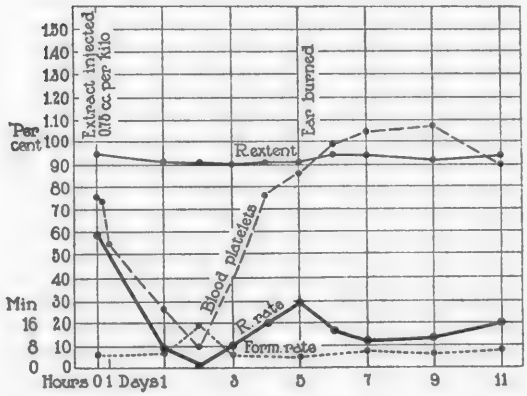
The pneumococcus extract of Avery and Neill (3) is known to have a specific lytic effect upon the blood platelets and to produce in mice a transitory purpuric condition. I am indebted to Dr. Avery and Dr. Julianelle of The Rockefeller Institute Hospital for a supply of the extract. This was first warmed to remove the hemolysin, and amounts from 0.25 to 3.50 cc. per kilo body weight were

[2] Heparin may be obtained from Hynson, Westcott and Dunning, of 150 Nassau Street, New York City, and Baltimore, Md.

injected intravenously in twelve rabbits. No symptoms were observed, although one rabbit receiving the highest dose was found dead after 18 hours. No purpura or tendency to bleed (as indicated by the incisions in the ear) was evident, even



TEXT-FIG. 7.



TEXT-FIG. 8.

when the platelets were practically absent from the blood stream. Microscopic study of the bone marrow, spleen, liver, lymph nodes and other organs and tissues discovered no changes. Charts of two of the rabbits are given (Text-figs. 7 and 8). Curves have been added representing the platelet content of the blood; these values

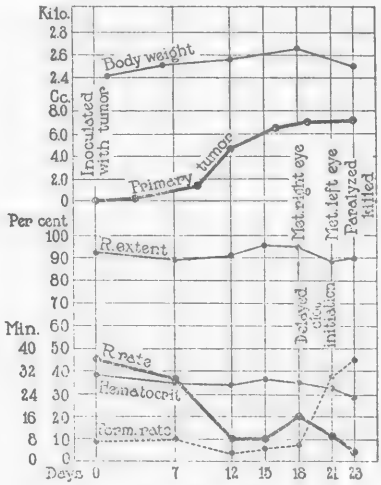
were obtained with the thrombocytocrit,[3] an instrument recently described (4), and give estimations of platelets volumetrically instead of numerically as customary. It will be seen that platelet lysis commenced immediately after injection of the extract and reached an extreme degree on the 2nd day. The platelets then reaccumulated rapidly and in great excess, attaining a maximum between the 7th and 9th days, after which their volume returned to normal. This behavior is similar to that described in the mouse by Julianelle and Reimann (5). In Text-fig. 7, following the injection of 1.25 cc. extract per kilo, the rate and extent of clot retraction began to fall within 1 hour, and continued to descend until the 2nd day, when the clot, although of firm consistency, failed to retract. The next day retraction had returned to normal in extent but took place at a very slow rate. The rate returned to its original level by the 7th day. Clot formation showed no departure from normal throughout. In Text-fig. 8, following the injection of a smaller dose, the retraction rate fell as before but its extent remained unaffected. By the 5th day the rate had begun to ascend, but once more was depressed coincidently with the occurrence of a slight accidental burn of the rabbit's ear while warming it to secure blood, and the typical reaction to the extract was thus altered. Clot formation became slightly retarded on the 2nd day. These changes in blood coagulation were found to vary in degree among the rabbits approximately with the size of dose employed. Even with as small an amount as 0.25 cc. per kilo retraction rate was markedly reduced, but the extent of retraction was affected only with the larger amounts of the extract. A close parallelism was demonstrated between retraction rate and the platelet content of the blood, even to the extent that thrombocytosis was associated with a slightly hypernormal retraction rate. The relation held, however, only in animals free from infections, for, as in Text-fig. 8, depression in rate took place under conditions of disease without regard for the platelet reading. The extent of retraction was interfered with only when the platelets reached an exceedingly low level.

II. Tumor.

The tumor which forms the basis of these experiments is an epithelioma of variable malignancy (6, 7). It arose spontaneously in a rabbit and has been carried in this species for purposes of study by successive transplantation. Testicular inoculation of tumor tissue, as routine, gives rise invariably to local neoplastic growth. Metastases develop in a high proportion of instances, often very profuse and resulting fatally within a period of from 20 to 80 days. In others, after a certain amount of growth, the neoplasm becomes necrotic and is absorbed, with survival of the animal.

[3] Obtainable from Arthur H. Thomas Co., Philadelphia, and from Firma Arno Haak, Jena, Germany.

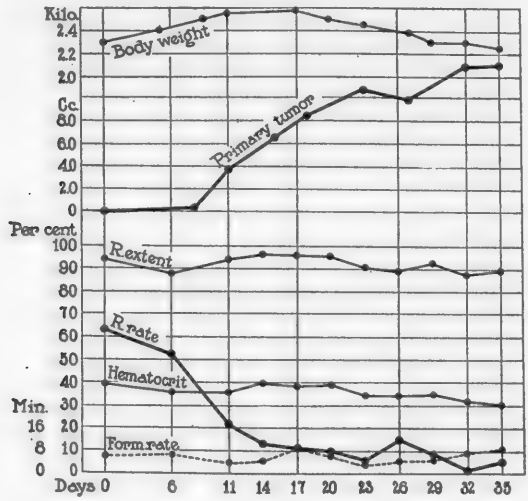
Examination of the blood coagulability has been carried out in 58 rabbits inoculated with tumor, tests being made at regular intervals throughout the course of the disease. In many cases the results have been difficult to interpret because of complication of the picture by the effects of spontaneous infections, most frequently appearing where the tumor ran a long and severe course and resulted in physical depletion of the animal. However, uncomplicated cases are abundantly at hand, and typical examples will be described.



TEXT-FIG. 9.

A very malignant form of the tumor is illustrated by an animal whose chart is given in Text-fig. 9. The primary tumor grew rapidly and on the 18th day a metastasis was discerned in the right iris. This developed vigorously and 3 days later the left eye was similarly involved. During this period the rabbit lost in strength and ate little. The hind quarters were found paralyzed on the 24th day and the experiment was terminated. At postmortem examination the general state of nourishment was found reduced but not depleted. The primary tumor measured 1.5 by 1.5 by 5.0 cm., had completely destroyed the testicle and invaded the spermatic cord. Extensive involvement by metastases of the abdominal viscera was presented, also, of both eyes, lymph nodes of the neck, femoral bone marrow on both sides and spinal column. The blood readings obtained are shown in the chart, a curve being added to represent the rate of growth of the primary

tumor as estimated by measurements during life (8). Shortly after inoculation, at a time when the initial tumor could be felt in the testicle as a pea-sized nodule, a distinct depression was present in clot retraction rate, and this value continued to fall still farther. On the 18th day the reading tended to return somewhat toward normal but soon fell again and reached a very low level at the end of the experiment. The curve of retraction rate, thus, is seen to represent practically an inversion of the growth curve of the tumor. Retraction extent remained within the normal range throughout. Clot initiation and formation were normal until the last 2 days when marked delay occurred in these functions.



TEXT-FIG. 10.

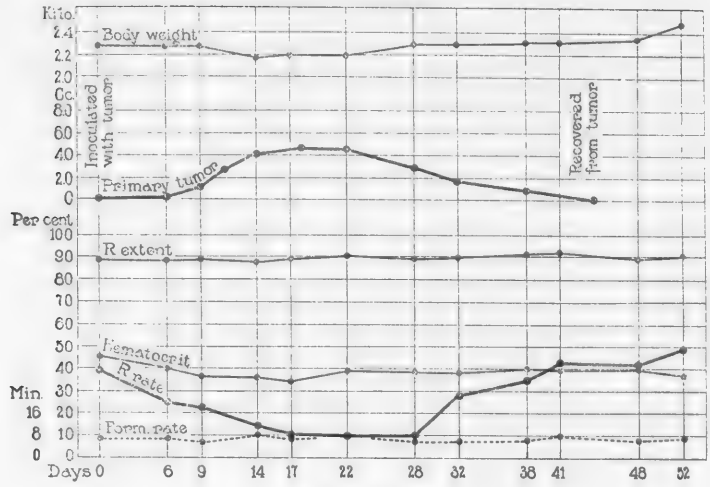
An instance of more gradually extending but severe neoplastic disease is given in Text-fig. 10. The primary tumor developed steadily to large size. On the 19th day the first metastasis was discovered and others followed. From this time on the animal lost in weight and by the 35th day had become emaciated and weak, when it was killed and autopsied. The body tissues were wasted. The primary tumor completely distended the scrotal sac, and tumor nodules were found in the spermatic cord and retroperitoneal lymphatics. The adrenals were largely replaced by metastases; the omentum contained clusters of them. Nodules were present also in the mediastinum, posterior lymphatics of the neck and right iris, and the nasal cavities and sinuses were filled with neoplastic growth. A nodule had entirely destroyed the hypophysis, eroding the sella turcica. In the chart it will be seen that clot retraction rate began to diminish at an early stage of tumor

development and dropped progressively with its further extension, to a low value at death. Here again, the curve of this function parallels inversely the tumor curve. Fluctuations which occurred in the extent of retraction and in clot formation rate were minor and lay within the limits of normal.

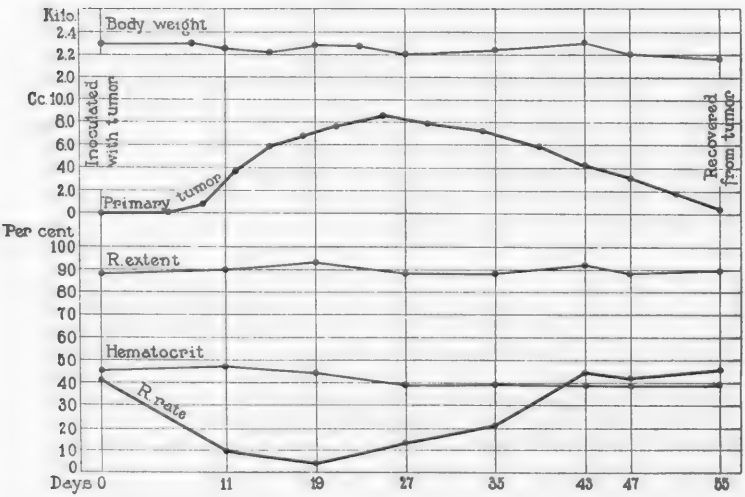
A comparatively benign course of the disease is illustrated in the cases of Text-figs. 11 and 12. The primary tumors grew to moderate (Text-fig. 11) or large (Text-fig. 12) size, and then receded without giving rise to discoverable metastases. The charts show a progressive depression of clot retraction rate during the early stages of the disease, and a return to normal as the tumors become absorbed. Retraction extent and formation rate showed no definite changes.

The rabbits whose charts are given in Text-figs. 13 and 14 are presented as types with complications. In the first, the testicular tumor developed to moderate proportions and metastases were felt in the abdominal wall on the 19th day. The iris of both eyes became involved. After the 25th day the tumors ceased to grow and were eventually absorbed. 48 days after inoculation an attack of snuffles set in with sneezing and profuse nasal discharge. A few days later the experiment was terminated. At postmortem examination the nasal cavities were found filled with pus; other than this and the scar of the healed primary tumor no lesions were present. The chart (Text-fig. 13) indicates that during the period of tumor activity retraction rate was depressed, and showed a secondary depression while the necrotic tumor tissue was being absorbed. After returning finally to a normal level, it dropped a third time at the onset of upper respiratory infection. Other factors in the blood tests were normal throughout.

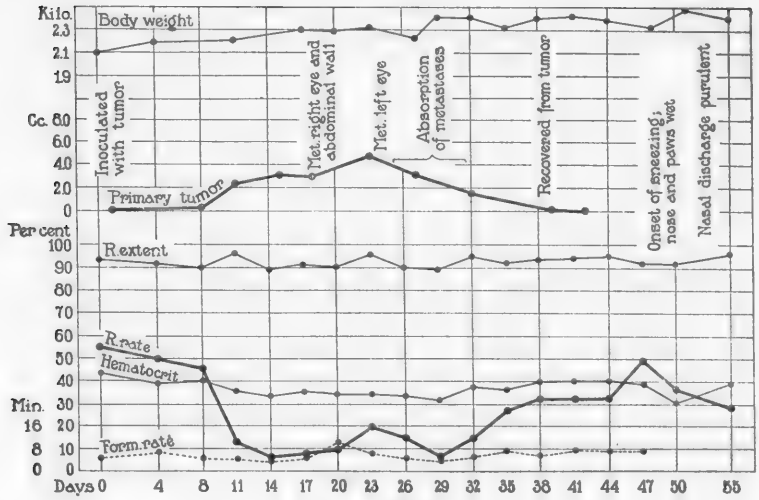
The rabbit of Text-fig. 14 pursued an extended and rather severe form of the disease. The testicle containing the primary tumor was withdrawn into the abdomen soon after inoculation and the growth of the tumor could not be followed. On the 28th day appeared a metastasis in the right iris and others followed in great numbers,—with masses of tumor to be felt in the deep tissues of the neck and in the abdomen. Growths developed at the gingival margins of the upper molar teeth, protruding into the mouth to such an extent as to interfere with eating. From the 30th day on the rabbit became thinner and ultimately cachectic, although it remained vigorous at all times. The experiment was terminated 58 days after inoculation. Autopsy: The tissues were everywhere wasted. An enormous primary tumor lay within the peritoneal cavity, and massive collections of metastases involved the retroperitoneal lymphatics, adrenals, kidneys, spleen, lungs, mediastinum, thymus, neck muscles and lymphatics, hypophysis, both eyes and the maxillary antrum, with infiltration of the alveolar processes and gums. All tumors, however, were thickly encapsulated and entirely necrotic. Evidently the neoplastic process had been arrested for some time, and the cachexia was the combined result of absorption of necrotic tumor tissue and the inability of the animal to take food properly. In the chart, retraction rate is indicated by a 1 hour serum reading in comparison with the final, and this value is seen to have fallen slightly during the period of metastasis (30th to 43rd days), and to have risen to a



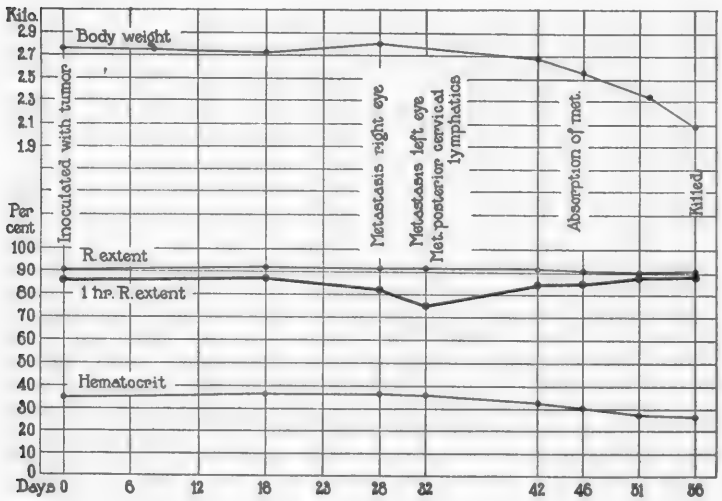
TEXT-FIG. 11.



TEXT-FIG. 12.



TEXT-FIG. 13.



TEXT-FIG. 14.

high position the last week of the experiment. Other factors in the tests showed no alterations of note.

DISCUSSION.

The reports in the literature are very inconsistent with regard to blood coagulability in disease in general, excluding the hemorrhagic diatheses, but where authors have used carefully controlled methods of testing the results indicate that coagulation rate and the retraction of the clot as observed qualitatively remain quite normal. Addis (9), in examining 112 patients, all of whom were seriously ill and presenting various sorts of disease both bacterial and non-bacterial in nature, found changes in coagulation rate in 30 per cent, and these were in an extremely critical condition when the blood was tested. Lee and White (10) conducting a similar investigation also obtained alterations only in the critically ill, and then not constantly. Pneumonia is an exception, for Dochez (11) and others have demonstrated consistent delay in clotting rate during its progressive stages; this is probably to be accounted for in the presence of the pneumococcus toxin (Avery) and its specific action upon elements of the blood connected with coagulation. Imperfectly retracting or non-retractile blood clot has been reported to occur occasionally in certain infections, *i.e.*, diphtheria, pneumonia, miliary tuberculosis, nephritis, ulcerative colitis, smallpox, etc., and these, too, have been cases of unusual severity. Thrombocytopenic purpura hæmorrhagica is the only condition which presents non-retractile blood clot with any regularity. The results of the present work in diseases of the rabbit agree with these views, *i.e.*, alterations in the rate of clot formation and extent of clot retraction were found confined to rapidly progressing pathological conditions, at the height of the disease and at terminal stages, and here inconstantly. They failed entirely to appear in gradually developing maladies, even where profound emaciation and weakness were reached.

One factor of the blood coagulative process, however, the rate with which the clot retracts, was found very sensitive to the presence of disease whether local or general in nature. Lesions without apparent constitutional effects, a small blistered burn, a sterile incision, a tumor of pin-head size, produced distinct fluctuations of this value. The

onset of rhinitis was indicated before the appearance of symptoms. The relation of clot retraction rate to the course of disease could be traced with particular assurity in the case of the tumor where a measure was also afforded of the progress of the lesion. Here, not only was the curve of retraction rate, in uncomplicated instances, nearly an exact reflection of the curve of tumor growth, but metastases were detected during or immediately following periods in the disease characterized by very low retraction rate readings. That reduction in the rate of clot retraction has to do with disease activity rather than with a poor state of bodily nutrition *per se*, is demonstrated by the fact that body weight was found to run parallel to retraction rate only in so far as it also reflected the stress of disease. Examples from the cases cited illustrate this: While in Text-fig. 11 body weight and clot retraction rate were simultaneously reduced during the active stages of tumor growth, in Text-figs. 9 and 10 the animals gained steadily in weight with the first inroads of the tumor, and retraction rate fell; in Text-fig. 14, when disease activity had totally ceased and cachexia was extreme, the rate of retraction remained at a normal level. There is also no consistent correspondence between anemia (hematocrit values) and defective clot retraction.

The alterations in blood composition that are responsible for changes in clot retraction have not been entirely explained. Hayem (12) showed that blood plasma deprived of all cellular elements formed a firm clot on standing and that the clot failed to retract. On the other hand, when he allowed the platelets alone to remain in the plasma, the clot which formed retracted normally. It was pointed out by Duke (13) that in purpura hæmorrhagica with a reduction in the number of platelets in the blood below about 50,000 per c.mm., specimens of blood formed non-retractile clots, and le Sourd and Pagniez (14), among others, have been able to induce clot retraction in such blood specimens by adding normal platelets. However, the latter authors (15) came to the conclusion, after both clinical and experimental observation, that, while the presence of blood platelets seems indispensable for normal retractility of the blood clot, retraction occasionally fails to take place even when platelets are present in abundance. This has been our experience, also, with regard to the relationship of both rate and extent of clot retraction to the blood

platelet content. It is possible also that the quality (fragility or chemical nature) of the platelets, as well as the quantity, may vary and play a rôle in clot retraction. The fact is well known, moreover, that the addition to the blood of minute traces of acid changes markedly the consistency of the clot, and the hydrogen ion concentration of the blood may readily be a determining factor in clot retractility.

SUMMARY.

Description is given of changes in blood coagulability found in diseases of the rabbit, including malignant tumor, spontaneous infections, non-bacterial diseases and lesions, and hemorrhagic states specifically induced. The changes involved variously the time of onset of blood coagulation, clot formation rate and the rate and extent of clot retraction.

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STUDIES ON THE OXIDATION AND REDUCTION OF IMMUNOLOGICAL SUBSTANCES.

V. Production of Antihemotoxin by Immunization with Oxidized Pneumococcus Hemotoxin.

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INTRODUCTION.

It is known that active or reduced pneumococcus hemotoxin is antigenic since immunization with it induces a so called "antihemotoxin" which neutralizes the lysin (1). There is a marked lessening in the hemolytic activity of oxidized pneumococcus extracts (2), and it has seemed important to determine whether a loss in antigenicity accompanies the inactivation of the hemolytic property. Our investigation of this question has consisted in a comparison of the immunity response to the injection of reduced and oxidized pneumococcus extracts.

EXPERIMENTAL.

Methods.

Substances Injected.—A series of rabbits were immunized by injection of the following substances: (1) "reduced" or active pneumococcus extract which was stored in sealed tubes and subjected to a minimum exposure to air before injection; (2) oxidized or inactive extract which was exposed to air for 6 hours at 37°C. and then stored at 5°C.; (3) "vaccine" or heat-killed suspensions of pneumococci.

The reduced extract contained the active hemotoxin in high concentration, 0.001 cc. producing hemolysis when added to 2.5 cc. of red blood cells. The oxidized extract contained inactive hemotoxin and before each injection was always tested for the presence of traces of active lysin. That no significant amount of it persisted in the oxidized extract was proved by the fact that 0.3 cc. of the extract caused no detectable hemolysis when added to 2.5 cc. of blood cells. The results

in animals injected with the heat-killed bacterial suspension were included for purposes of comparative study. The bacterial suspensions were heated at 56°C. for 30 minutes. Young cultures were used and the suspensions were heated at once to minimize autolysis and cell solution.

Immunization.—The reduced and the oxidized sterile cell solutions were injected into rabbits intravenously on 6 consecutive days, followed by a free interval of 1 week. Four courses of injections were given, the doses gradually increasing from 0.1 cc. to a maximum of 2.0 cc., in the last series of injections.

The "vaccine" or heat-killed bacterial suspension was also injected intravenously at the same time as the sterile cell solutions. Four courses of six daily injections of 0.1 cc. of a concentrated suspension of the bacteria were given so that the animals received in all the equivalent of bacteria from 24 cc. of culture.

All of the rabbits were bled on the 10th day after the last injection.

Titration of Antihemotoxin in Immune Serum.—The method used in determining the antihemotoxin value of the immune serum consisted in adding various amounts of serum to a constant amount of hemotoxin and determining the amount of serum required to completely neutralize the lysin. The hemotoxin-serum mixtures were shaken and then incubated for 45 minutes to allow time for combination of hemotoxin and its neutralizing antibody; a constant amount of blood cells was then added and the final test systems incubated to determine the presence of free or unneutralized hemotoxin. The detailed procedure is given below:

Constant "Dose" of Hemotoxin.—The hemotoxin content of a sterile pneumococcus extract was determined by a preliminary titration. Three times the amount of extract found sufficient to completely hemolyze 2.5 cc. of a 1 per cent suspension of washed rabbit cells was chosen as the test "dose" of hemotoxin. The pneumococcus extract was then diluted to such a concentration that 1.5 cc. of the dilute solution contained this amount of lysin.

Hemotoxin-Serum Mixtures.—0.5 cc. of different dilutions of serum was added to the series of tubes containing 1.5 cc. of the dilute hemotoxin solution. Owing to the inhibitory effect on the lysin of certain constituents of normal serum, control tubes were included containing the sera of the animals from bleedings made before immunization. The mixtures of hemotoxin and serum were shaken and then incubated for 45 minutes at 37°C.

Final Hemolysis Tests.—At the end of the 45 minutes, the presence of free or unneutralized lysin was determined by adding 0.5 cc. of a 5 per cent suspension of rabbit cells to the hemotoxin-serum mixtures. These final hemolysis test mixtures were incubated for 1 hour and readings of hemolysis made after centrifugation.

Control of Lysin "Deterioration" during Incubation of Hemotoxin-Serum Mixtures.—It was necessary to rule out the possibility that the lysin might be inactivated by oxidation since, should this occur, it might be confused with the immunological neutralization. For this purpose, control mixtures of 0.5 cc. salt solution plus 1.5 cc. of dilute hemotoxin were incubated with the series. At the end of the incubation period, these mixtures were diluted one-half and one-third; and the usual amount of red blood cells was added after these dilutions of the

control lysin had been made up to the correct volume of 2.0 cc. The control test mixture containing one-half and one-third the amount of lysin used, always showed complete hemolysis which proves that none of the apparent lysin neutralization or inhibition can have been due to inactivation of hemotoxin by oxidation during the preliminary incubation period.

Absence of Visible Precipitation in the Incubated Hemotoxin-Serum Mixtures.—Titrations of immune serum for the presence of antibodies neutralizing hemotoxins or enzymes are often complicated by the fact that the tested sera contain precipitins for other proteins present in the cell solutions. If during the preliminary incubation of the serum and cell solution, a precipitation or flocculation occurs, it is always possible that the active substance may be carried down with the precipitated particles. Subsequent tests of mixtures in which this precipitation has occurred indicate an apparent inhibition or neutralization of the test substance although in fact the active substance may have been only mechanically removed.

No such source of error existed in our experiments; for there was never any visible clouding or precipitation. The point is important since the immune sera contain a precipitin for the pneumococcus protein present in the bacterial cell solution used to furnish the hemotoxin. When the serum and bacterial solution are less dilute than in mixtures made according to the above procedure, protein precipitation occurs.

Strength of the Antihemotoxin in the Serum of Animals Immunized with Oxidized (Inactive) and Reduced (Active) Pneumococcus Hemotoxin.

In the following experiment a comparison was made of the hemotoxin neutralizing power of the sera of animals immunized with oxidized (inactive) hemotoxin and that of sera immunized with the reduced (active) hemotoxin. The serum of a rabbit immunized with a heat-killed suspension of intact pneumococci was included for comparison. The general procedure has been described under Methods. In this experiment, the immune sera were tested against hemotoxin derived from pneumococci of the same fixed type (Type II) as the cell solutions used for immunization. The results are given in Table I.

The marked neutralizing power of the sera of animals immunized with both the oxidized (inactive) and the reduced (active) solutions of pneumococci is evident in Table I. The production of an antihemotoxin by the injection of cell solutions containing the active hemotoxin has been shown in Cole's (1) studies. The new fact revealed in Table I is the production of a neutralizing antibody by immunization with oxidized, that is to say, inactive pneumococcus hemotoxin. It is

evident that the serum of the animals injected with an oxidized extract which contains no demonstrable active hemotoxin possesses approximately the same neutralizing action as does that of animals immunized with the reduced, or active, hemotoxin.

The animal immunized with the heat-killed suspension did not produce antihemotoxin. This is an interesting correlation with the similar failure of immunization with properly prepared "vaccines" to yield an antibody for pneumococcus protein which like the hemotoxin is an endocellular substance (4).

TABLE I.

Antihemotoxin in Sera of Animals Immunized with Reduced (Active) Hemotoxin and Oxidized (Inactive) Hemotoxin.

No. of rabbit	Immunized by injection with	Hemolysis by previously incubated mixtures of hemotoxin and serum						
		Immune serum					Normal serum, before immunization	
		Amount of serum					Amount of serum	
		0.01 cc.	0.006 cc.	0.003 cc.	0.002 cc.	0.001 cc.	0.05 cc.	0.01 cc.
1	Inactive hemotoxin in oxidized cell solution	0	0	0	0	0	++++	++++
2	" "	0	0	0	+	±	+++	++++
3	Active hemotoxin in reduced cell solution	0	0	0	0	±	+++	++++
4	" "	0	0	0	0	0	+++	++++
5	"Vaccine" (heat-killed suspension of intact cells)	+++	++++	++++	++++	++++	++++	++++

Species Specificity of the Antihemotoxin Produced by Immunization with Oxidized (Inactive) Pneumococcus Hemotoxin.

In the preceding experiment, the neutralizing capacity of the immune sera was tested against hemotoxin derived from the homologous type of pneumococci. Cole (1) has shown that the antihemotoxin produced by immunization with the active substance is not type-specific,

but is effective against hemotoxin derived from all types of pneumococci. It seemed of interest to determine if the antihemotoxin produced by immunization with inactive (oxidized) hemotoxin is likewise equally effective against the hemotoxin derived from heterologous types of pneumococci. The protocol of an experiment testing this question is presented in Table II.

As shown in Table II the antihemotoxin which is produced by immunization with oxidized or inactive Pneumococcus is only species-

TABLE II.

Species Specificity of the Antihemotoxin Produced by Immunization with Oxidized (Inactive) Pneumococcus Hemotoxin.

Serum of animal immunized against	Hemolysis by previously incubated mixtures of hemotoxin and serum					
	Hemotoxin from Type I Pneumococcus		Hemotoxin from Type II Pneumococcus		Hemotoxin from Type III Pneumococcus	
	Amount of serum		Amount of serum		Amount of serum	
	0.01 cc.	0.002 cc.	0.01 cc.	0.002 cc.	0.01 cc.	0.002 cc.
Inactive hemotoxin in oxidized pneumococcus cell solution (Type II)	0	0	0	0	0	0
Active hemotoxin in reduced pneumococcus cell solutions (Type II)	0	0	0	0	0	0
"Vaccine" (heat-killed suspension of intact cells)	++++	++++	++++	++++	++++	++++

specific and not type-specific since it neutralizes the hemotoxin derived from the cells of heterologous types of pneumococci. A species-specific antibody is apparently the usual type involved in the neutralization of bacterial hemotoxins; for the hemotoxin from cholera vibrios which are type-specific by agglutination, likewise gives rise to a common neutralizing antibody (3). The same holds true of the neutralizing antibody for the true toxins formed by diphtheria, tetanus and Welch bacilli, although each of these bacterial species includes strains which exhibit type specificity in tests with agglutinating immune sera.

Comparison of the Inhibiting Effect of Normal and Immune Serum upon Digitonin Hemolysis.

The serum of normal animals possesses a certain inhibitory action upon pneumococcus hemotoxin. At least a part of this inhibition by normal or non-immune serum can be referred to lipoid constituents. In the case of tetanolysin, a number of investigations have proved that the increase in the neutralizing action of immune serum is not due to an increased concentration of the lysin-inhibiting constituents of normal serum, and it might be concluded by analogy that this is also true for the pneumococcus hemotoxin. However, in view of the fact that the antihemotoxin described in the present paper is produced by immunization with a non-hemolytic antigen, it seemed desirable to determine whether the increased neutralizing capacity is due to a non-specific substance which would inhibit the hemolytic activity of other lytic agents.

Digitonin was chosen as the lytic substance to test this question, largely because hemolytically active digitonin is known to combine with cholesterol to form a non-hemolytic compound. This reaction, in fact, is used as the basis of certain methods employed in the quantitative estimation of the cholesterol content of normal serum (4). The combination of cholesterol and digitonin is especially suited to a comparison of the degrees of neutralization of pneumococcus hemotoxin by normal and immune sera since Cole (1) has shown cholesterol to be one of the most important constituents involved in the inhibitory action of normal serum upon the pneumococcus lysin.

The experiments may be outlined as follows: The lytic action of a sample of digitonin was determined in the absence of serum. With this information at hand two dilutions of digitonin were prepared in salt solution; one solution contained in 1.5 cc. twice the amount required to hemolyze 2.5 cc. of a 1 per cent suspension of rabbit cells; the other solution contained four times that amount of the lytic agent. Mixtures of the digitonin with various dilutions of normal and immune rabbit serum were then prepared as described for the test mixture of pneumococcus hemotoxin and serum in the previous experiments. After incubation for 1 hour at 37°C. to allow time for the combination of digitonin with the cholesterol and other serum constituents, 0.5 cc. of a 5 per cent suspension of rabbit cells was added.

The results of these experiments were conclusive. Although relatively large amounts of both the normal and immune serum inhibited the digitonin hemolysis, there was no significant difference in the action of the two. The sera obtained by immunization with both the reduced and oxidized solutions exhibit more than 100 times the neutralizing capacity of normal serum when tested against pneumococcus hemotoxin, yet they exhibited no greater neutralizing or inhibiting action on digitonin hemolysis than did this serum. From the results, it is obvious that the neutralizing antibody produced during immunization with the hemolytically inactive, oxidized hemotoxin is distinct from the usual lysin-inhibiting constituents of normal serum.

Neutralization Experiments with Pneumococcus Immune Serum and Hemotoxins of Other Bacteria.

Experiments were conducted to determine whether the immune sera which neutralize pneumococcus hemotoxin have any neutralizing or inhibiting action upon the hemotoxins of other bacteria. Tetanolysin and the lysin of the Welch bacillus were the hemotoxins tested. Normal serum and the serum produced by immunization with heat-killed pneumococcus vaccine were included as controls, since certain constituents of normal serum have a marked inhibitory action upon the lysins of both of these anaerobic bacilli. The procedure employed was essentially the same as in the previous experiments with the pneumococcus lysin. The constant "dose" of tetanolysin and Welch lysin used in these experiments represents 8 to 10 times the amount of lysin required for the hemolysis of 2.5 cc. of rabbit cells in the absence of serum. A larger "dose" of lysin was chosen than in the previous experiments because these lysins are inhibited to a greater extent by normal serum than is the lysin of pneumococci. The preparation of serum-hemotoxin mixtures and other steps in the procedure were the same previously described.

Three normal sera and the sera of the four rabbits immunized with oxidized and with reduced pneumococcus extracts were included in the experiment. The results of tests upon tetanolysin with a typical normal and a typical immune serum are presented in Table III.

In spite of the markedly increased capacity of the immune serum to

neutralize the hemotoxin of Pneumococcus, the results presented in Table III which were typical for the experiments show that immunization of animals with this hemotoxin does not increase the ability of their serum to neutralize or inhibit the hemotoxin of the tetanus bacilli.

The results of the tests with the Welch lysin were not so clean-cut, since they were complicated by marked differences in the neutralizing or inhibiting capacity of different normal rabbit sera—a fact previously observed in another investigation (5). The sera of the various immune rabbits also showed marked differences among themselves in their inhibitory action on the Welch lysin. However, a survey of the results as a whole indicates quite clearly that the sera of rabbits

TABLE III.

Inhibition or Neutralization of Tetanolysin by Pneumococcus Immune Serum and by Normal Serum.

Serum	Hemolysis by previously incubated mixtures of serum and tetanolysin		
	Amount of serum		
	0.005 cc.	0.001 cc.	0.0002 cc.
Rabbit immunized against pneumococcus hemotoxin	0	+	+++
Normal rabbit	0	0	++

immunized against the hemotoxin of Pneumococcus exerted no greater neutralizing or inhibiting effect upon the lysin of Welch bacilli than was found with the serum of normal or non-immune animals.

COMMENT.

The preceding experiments indicate that a neutralizing antibody may be produced by immunization with the hemolytically inactive hemotoxin present in oxidized solutions as well as by immunization with the active hemotoxin. The antibody is a species-specific antihemotoxin neutralizing the hemotoxin from all types of pneumococci. It seems to be without effect upon the hemotoxins of tetanus and Welch bacilli.

The theoretical and practical importance of "toxoid immunization"

gives a special significance to the above results. But the data thus far procured do not enable one to state that the inactive or oxidized lysin possesses antigenic properties identical with those of the reduced or active hemotoxin. A more complete discussion of the antigenic properties of the inactive oxidation products of pneumococcus hemotoxin will be presented in a subsequent paper.

SUMMARY.

Immunization with solutions of the intracellular substances of pneumococci, in which the hemotoxin has been rendered inactive by oxidation, yields an antibody which neutralizes the active or reduced hemotoxin.

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EXPERIMENTAL OBSERVATIONS ON IRRADIATED, NORMAL, AND PARTIALLY PARATHYROIDECTOMIZED RABBITS.

I. The Effects of Partial Parathyroidectomy.

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Observations on the endocrine tissues of rabbits irradiated with a quartz mercury arc lamp have disclosed the striking hypertrophy of the parathyroid glands produced by the irradiation (1). Histologically, this hypertrophy was found to be a true hyperplasia of the endocrine tissue, but a study of the calcium and inorganic phosphate content of the blood of these rabbits failed to reveal any significant changes from the normal levels (2).

That parathyroid integrity is essential to the maintenance of a normal blood calcium level has been established by the extirpation experiments of many investigators, but the upper limit of calcium concentration seems to be determined by factors independent of parathyroid control. Hence, in the normal animal, parathyroid hypertrophy due to an external stimulus, such as light, appears to result only in an increase in the factor of safety, and to be unaccompanied by a recognizable increase in functional activity for which there is no physiological demand.

On the other hand, the presence of an increased factor of safety might be revealed by an unusual functional strain or emergency, and a study of such conditions, experimentally produced, might throw further light upon normal parathyroid function. Having at hand methods of producing a hypertrophic condition of the parathyroid glands by means of ultra-violet light, and a condition of reduced function by partial extirpation, we undertook a series of experimental comparisons of groups of rabbits with enlarged, normal, or partially extirpated parathyroids.

In preparation for these studies, partial parathyroidectomies were performed on a number of rabbits, some normal, some after irradiation with ultra-violet light. The effects of the operation itself upon the calcium and the inorganic phosphate level of the blood of these animals form the subject matter of this paper.

Experimental Procedure.

Since the partially parathyroidectomized rabbits were to be used subsequently in other experiments, the operations were performed on small groups of animals and at various seasons of the year. In some instances only the two external parathyroids were removed; in others, the thyroid gland was divided at the isthmus, and one lobe, with its internal parathyroid gland, was removed also. The animals were all healthy, adult, albino males, and the glands were removed aseptically, under ether anesthesia, after a median incision and blunt dissection of the tissues of the neck. The operative wounds healed without infection. At intervals after the operation, small samples of blood were taken from the marginal ear vein and allowed to clot, and calcium (3) and inorganic phosphorus (4) determinations were made on the serum.

Preliminary observations on two normal rabbits, after removal of the two external parathyroid glands, showed that the operation was followed by a sharp drop in blood calcium to a low level on the 2nd or 3rd day, and a more gradual increase toward the normal over a period of days or even weeks. No signs of tetany appeared in these two animals.

Experiment 1.—The next groups of six and four normal rabbits respectively were deprived of both external parathyroid glands and one internal gland also. In spite of individual variations in reaction, the trends of the blood calcium curves of these rabbits were essentially similar. Immediately after operation, the blood calcium of all ten rabbits dropped from normal levels, between 11.6 and 13.0 mg. per 100 cc. of serum, to 6.2 to 10.2 mg. (37 to 88 per cent of the control figures) by the 2nd day. The rabbit with 6.2 mg. of calcium per 100 cc. of blood serum succumbed on that day in severe tetany. From this point the blood calcium of seven of the remaining nine rabbits began to rise towards normal, so that figures of 10.8 mg. to 12.6 mg. were obtained on the 4th to the 6th days, and these levels were usually maintained thereafter (Chart 1). The other two rabbits had persistently lower blood calcium levels, and one of them showed a drop from 8.5 mg. per 100 cc. of serum on the 14th day after operation, to 4.8 mg. on the 17th day, when it had a typical tetanic seizure and died. The other rabbit maintained a serum calcium level between 8 and 10 mg. for 35 days. 2 days later it was transferred to a fasting

experiment, in which it succumbed on the 5th day. On the day before death its blood calcium was 5.65 mg. per 100 cc. of serum.

The inorganic phosphorus in the blood serum of most of these rabbits was not examined at regular intervals. It was observed, however, that before death the serum phosphorus of the rabbits that succumbed on the 2nd, 17th, and 42nd days after operation was 9.4 mg., 13.1 mg., and 12.0 mg. respectively per 100 cc. of serum, as compared with a normal average of 7.4 mg.

In this experiment the capacity of the residual parathyroid tissue to respond to sudden demands is shown by the fact that in seven of ten rabbits, after the initial drop, blood calcium levels only 5 to 10 per cent below normal were attained within a week, and these levels were usually maintained thereafter. If, after partial para-

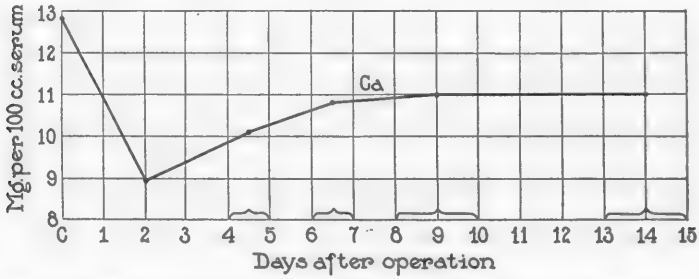

CHART 1. Experiment 1. Serum Ca levels after partial parathyroidectomy.

thyroidectomy in normal rabbits, the remaining tissue is usually able to restore the blood calcium level nearly to normal in 4 to 6 days, this is the interval in which evidence of parathyroid hyperplasia and consequent increase in the factor of safety must be sought in irradiated rabbits.

In the following experiment, normal and irradiated rabbits were partially parathyroidectomized and observed during the ensuing period.

Experiment 2.—A group of ten adult albino rabbits had both external parathyroids removed. Five of these animals, with backs clipped and ears shaved, had been exposed for 30 minutes a day, 6 days a week, for 3 weeks, at 1 meter distance, to a quartz mercury arc lamp (67 volts, 5.5 amperes). These rabbits presumably had hypertrophic parathyroid glands (1). The exposures were continued for the further period of observation. The five other rabbits served as controls for the

irradiated group. All were bled for a serum sample before operation, on the 1st, 2nd, and 4th days following, and then every 2 or 3 days to the 15th or 16th day.

A difference in the reaction of the irradiated and control rabbits to the loss of their external parathyroids became apparent within 24 hours after operation. None of the irradiated rabbits showed signs of tetany, but three of the controls were observed in acute attacks, characterized by gross and fibrillar twitchings of the voluntary muscles,—notably those of the jaw,—tonic and clonic convulsions, opisthotonos, deep, heaving respiration, venous engorgement, and

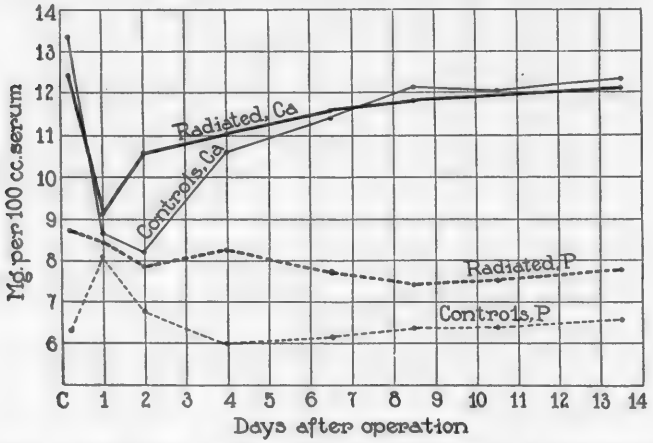

CHART 2. Experiment 2. Comparison of serum Ca and P in irradiated and control rabbits after partial parathyroidectomy.

excessive salivation. In two of these rabbits an intravenous injection of 10 cc. of $\frac{M}{8}$ $CaCl_2$ relieved the signs immediately, but one of them succumbed during the following night. The third rabbit recovered spontaneously.

The absence of all signs of tetany in the five irradiated rabbits led us to infer that the hyperplasia of the internal glands produced by the radiations had increased their functional capacity, *i.e.*, their factor of safety, to a degree sufficient to protect the animals. This increase in the factor of safety in the irradiated rabbits is reflected in a comparison of their blood calcium and inorganic phosphorus

curves. In both groups the fall in calcium was immediate, so that 24 hours after operation the average for the irradiated rabbits had dropped from 12.4 to 9.1 mg. (73 per cent of normal), and for the controls from 13.4 to 8.7 mg. (65 per cent of normal) per 100 cc. of serum. But the following day the blood calcium average in the irradiated group rose to 10.6 mg. per 100 cc. of serum, while the four survivors of the control group suffered a further loss to 8.2 mg. By the 7th day this difference had been overcome, and both groups then maintained calcium levels approaching normal (Chart 2). Similarly, due to sharp rises in the blood phosphorus of the three control rabbits

TABLE I.

Blood Calcium and Inorganic Phosphorus 1 Day after Partial Parathyroidectomy.

Normal rabbits				Irradiated rabbits			
No.	Calcium	Phosphorus	Ratio $\frac{Ca}{P}$	No.	Calcium	Phosphorus	Ratio $\frac{Ca}{P}$
	Mg. per 100 cc. serum				Mg. per 100 cc. serum		
18	10.9	6.0	1.82	13	10.3	8.5	1.21
19	9.5	4.8	1.96	14	10.1	7.2	1.40
20*	8.5	8.7	.98	15	8.9	8.6	1.03
21†	8.0	11.0	.73	16	8.5	8.7	.98
22*	6.0	9.9	.61	17	7.8	9.2	.85

* Observed in tetany.
† Died.

that had outspoken tetany, the average for the group was carried upward on the 1st day from 6.3 mg. to 8.1 mg. per 100 cc. of serum. Meanwhile, in the irradiated group, with a high average blood phosphorus to start with (2), only one of the rabbits showed a further rise in blood phosphorus following the operation. This rabbit had the greatest drop in calcium of any in the irradiated group.

Such a reciprocal relationship between blood calcium and phosphorus, pointed out by Howland and Kramer (5), and recently observed in normal dogs by Mayerson, Gunther, and Laurens (6), is illustrated in this experiment by arranging the rabbits of each group in the order of their blood calcium levels on the day following partial parathyroidectomy (Table I). In both groups the rabbits

with the lower calcium values after partial parathyroidectomy had the higher figures for blood phosphorus.

A final group of five normal rabbits, whose external parathyroid glands were extirpated, confirmed the earlier observations by exhibiting an average fall of blood calcium from 12.0 mg. to 8.8 mg. per 100 cc. of serum, 24 hours after operation. In three of these rabbits the calcium level was restored to normal in 7 to 9 days. The other two had persistently lowered blood calcium, and one rabbit, after transfer to a fasting experiment, on the 9th day, suffered a further drop in Ca to 4.9 mg. and died on the 16th day after the partial parathyroidectomy. 2 days before death this rabbit's blood

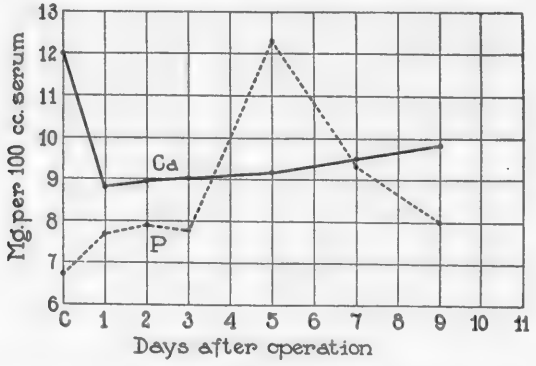

CHART 3. Serum Ca and P levels after partial parathyroidectomy.

phosphorus was 12.68 mg. per 100 cc. of serum. All the rabbits in this group exhibited a high blood phosphorus at one time or another after operation, the average for the group on the 5th day being 12.3 mg. per 100 cc. of serum, compared with a control level before operation of 6.7 mg. (Chart 3).

That the ether anesthesia and the operative procedure did not contribute to the fall in serum calcium, or the rise of inorganic phosphorus, was shown by the absence of such changes in two control rabbits, in which identical operations were performed, except for the removal of the parathyroid glands.

The Relative Significance of a Fall in Blood Calcium and of a Rise in Blood Phosphorus in Parathyroid Tetany.

We have already noted the marked rise in the inorganic phosphorus of the blood serum, which often accompanied the drop in serum calcium after partial parathyroidectomy. A rise in inorganic phosphorus occurred in some rabbits which did not develop tetany, but it was never absent in those that did, and was apparently associated with the onset of the acute attack. The question arises whether it is only the fall in calcium, or in reality the accompanying change in the ratio of calcium to phosphorus in the blood serum that is more significant in tetany.

TABLE II.

Rabbit No.	Onset of tetany day after operation	Ca	P	Ratio $\frac{Ca}{P}$	Outcome
		Mg. per 100 cc. serum			
4	17th	4.8	13.1	.37	Died
6	41st	5.7	12.0	.48	Died
9	2nd	6.2	9.4	.66	Died
21	1st	7.9	11.0	.72	Given $CaCl_2$; died
20	1st	8.5	8.7	.98	Mild attack; recovered
22	1st	6.0	9.9	.61	Given $CaCl_2$; recovered
26	16th	4.9	12.7	.38	Died
Average.....		6.3	11.0	.60	

Normally, in rabbits, this ratio $\frac{Ca}{P}$ is not less than 1, and it usually lies between 1.3 and 2.6. Among 103 normal rabbits, with a calcium level, on admission, between 11.1 and 13.9 mg. per 100 cc. of serum, the lowest ratio $\frac{Ca}{P}$ was 1.10 and the three highest, with one exception, were 3.3. The exceptional rabbit had a very low serum phosphorus (1.8 mg.) and a unique ratio $\frac{Ca}{P}$ of 7.30. The average ratio for all the rabbits was $\frac{12.4}{6.4} = 1.9$.

In the rabbits that developed acute tetany after partial parathy-

roidectomy, on the other hand, the ratio $\frac{Ca}{P}$ was changed not only by the fall in calcium, but by an abnormal rise in phosphorus also. Table II shows the ratio $\frac{Ca}{P}$ in these rabbits, according to the last figures obtained before the attack, and it will be noted that high phosphorus figures contribute as much to change the $\frac{Ca}{P}$ ratio from normal, as do the low figures for blood calcium.

Of nine other partially parathyroidectomized rabbits, which were not observed in tetany, four at one time or another had calcium

TABLE III.

Rabbit No.	No. of days after operation	Lowest calcium level	Phosphorus level	Ratio $\frac{Ca}{P}$
		Mg. per 100 cc. serum		
3	2	8.6	7.7	1.12
5	2	7.6	8.6	.88
7	18	10.6	5.7	1.9
19	2	8.4	5.6	1.5
18	1	10.9	6.0	1.82
8	1	9.2	8.0	1.15
10	1	9.6	10.8	.89
11	5	7.3	12.2	.60
12	3	6.6	8.1	.81

and phosphorus levels comparable with some of those in the tetanic animals. The other five did not have either very low calcium or high phosphorus at any time after operation, and their $\frac{Ca}{P}$ ratios were usually more than 1 (Table III).

DISCUSSION.

Our observations are in accord with those of other investigators who have studied calcium and phosphorus metabolism after parathyroidectomy. The characteristic drop in blood calcium has often been found and needs no further comment. This fall in blood calcium is certainly a primary reaction, which may or may not be followed by a

rise in phosphorus, either immediately, or later, when the blood calcium is returning toward the normal level.

A phosphorus retention, following parathyroidectomy in dogs, was observed in 1911 by Greenwald (7), who has since published a series of papers in which the work of others is reviewed. He has recently stated (8, *a*) that in over 50 parathyroidectomized dogs tetany never appeared without phosphorus retention, and conversely, a drop in phosphorus excretion after attempted parathyroidectomy was observed in only one of ten dogs that did not develop tetany. However, the increase in "acid-soluble" phosphorus in the blood of parathyroidectomized dogs in tetany, which Greenwald had observed in 1913 (8, *b*), was not so marked in his later experiments, and in 1924 (8, *c*) he appears to minimize its significance, assuming that the excess of phosphates retained is deposited in the tissues, and only reappears in the circulation under certain abnormal conditions, such as after ether anesthesia, or cerebral or general anemia. In those of our rabbits that developed tetany, an excess of phosphorus was found in the blood.

Salvesen (9) also found that the inorganic phosphorus of the blood serum of completely parathyroidectomized dogs was considerably increased, and tended to remain above the normal value. According to one protocol, a dog with a normal blood calcium of 10 mg. per 100 cc. of serum and inorganic phosphorus of 3.0 mg. $\left(\frac{Ca}{P} = 3.3\right)$ developed severe tetany 2 days after complete parathyroidectomy, with a $\frac{Ca}{P}$ ratio of $\frac{3.50}{12.5} = 0.28$, and the serum phosphorus level remained above 6.0 mg. per 100 cc. of serum thereafter.

In a study on blood changes and clinical symptoms, following oral administration of phosphates in dogs, Salvesen, Hastings, and McIntosh (10) discuss the changes in blood phosphorus and calcium that result, and they conclude that the fall in calcium is the significant factor in the development of tetany.

In a recent private communication, Dr. John Howland stated his conviction that the clinical syndrome known as infantile tetany is certainly not the result of a high phosphorus content of the serum which drives down the calcium concentration (11), and observed that infantile tetany is found at times with a very low phosphorus concentration of serum. He has found that in many instances the phosphorus is high, perhaps in two-thirds of the cases, but there is the one-third which cannot be neglected.

We shall postpone further discussion of the subject until we report a series of observations on rabbits to which secondary sodium orthophosphate was given subcutaneously. The point to be noted at this time is that the calcium level itself was apparently not the sole determining factor in the development of tetanic signs in these partially

parathyroidectomized rabbits. Only in connection with an increase in blood phosphorus, which further disturbed the normal $\frac{Ca}{P}$ ratio, were signs of hyperirritability observed.

SUMMARY.

Previous irradiation of rabbits with ultra-violet light, with a consequent hypertrophy of the parathyroid glands, resulted in some degree of protection to these animals, when part of the parathyroid tissue was extirpated. This fact indicates that the remaining hyperplastic tissue was potentially active, and that the increased factor of safety represented by this condition of the remaining tissue resulted in less loss of blood calcium, a more rapid initiation of recovery, and an absence of the rise in the inorganic phosphorus of the blood serum which was a characteristic result of partial parathyroidectomy in normal rabbits. A fall in calcium in all the irradiated rabbits, without a corresponding rise in phosphorus, shows that the drop in calcium is the primary reaction, and a rise in phosphorus a secondary reaction following parathyroidectomy. But the relation of the rise in phosphorus to the development of tetany remains obscure.

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EXPERIMENTAL OBSERVATIONS ON IRRADIATED, NORMAL, AND PARTIALLY PARATHYROIDECTOMIZED RABBITS.

II. The Effects of Injections of Calcium Chloride or of Disodium Hydrogen Phosphate.

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Although the calcium and inorganic phosphorus levels of the blood serum of rabbits appear to be more affected by seasonal changes in environment and by caging (1) than by the hypertrophy of the parathyroid glands induced by ultra-violet radiations (2), the hypertrophic changes in the glands are evidence of an increased "factor of safety" which is manifest in the reactions of irradiated animals to partial parathyroidectomy (3). A comparison of their behavior with that of normal rabbits subjected to an identical operation[1] led us to a further investigation of the reactions of irradiated, normal, and partially parathyroidectomized rabbits to other experimental procedures, in order to test the functional capacity of their parathyroids. The three groups of rabbits were assumed to differ in the relative amount and potential activity of their parathyroid tissue, the irradiated animals having hypertrophic glands and the partially parathyroidectomized rabbits having only so much parathyroid tissue as was necessary for the maintenance of a blood calcium level approaching normal.

Our first observations on the effects of the injection of calcium chloride or of disodium hydrogen phosphate into irradiated, normal, and partially parathyroidectomized rabbits gave some unexpected results, and turned our immediate attention from the primary object of the investigation to an interest in the rôle of inorganic phos-

[1] All operations for removal of parathyroids were performed under full ether anesthesia.

phates in the development of outspoken clinical tetany, under conditions of lowered blood calcium.

Calcium Injections.

Two experiments were performed, the first with four rabbits; the second with seven.

Experiment 1.—Two normal, adult, male rabbits served as controls. The other two, with backs and ears closely clipped, had been exposed daily for 30 days to the

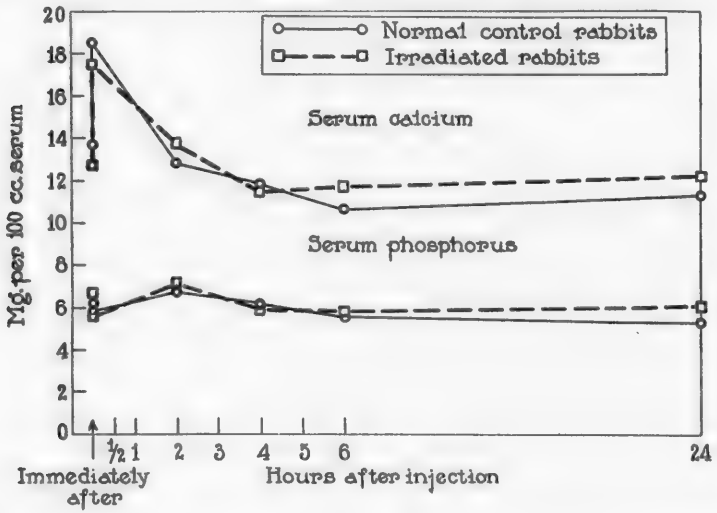

CHART 1. Experiment 1. Serum Ca after injection of $CaCl_2$ in normal and irradiated rabbits.

total radiations of a quartz mercury arc lamp (67 volts, 5.5 amperes), at 1 meter's distance. During this period the daily exposures were increased from 10 minutes to 2 hours. Then all four rabbits received an intravenous injection of sterile $\frac{M}{8}$ $CaCl_2$ solution, representing 25 mg. of Ca per kilo body weight. The volume of fluid was about 10 cc. Blood samples were taken before and immediately after the injections, and 2, 4, 6, and 24 hours later, and calcium (4) and inorganic phosphorus (5) determinations were made on the serum.

Chart 1 shows that the reactions of the normal and the irradiated rabbits were essentially similar. The excess calcium practically

disappeared from the blood in less than 2 hours, and the calcium level subsequently fell somewhat below that of the control specimens. This dose of calcium chloride had only a relatively slight effect upon

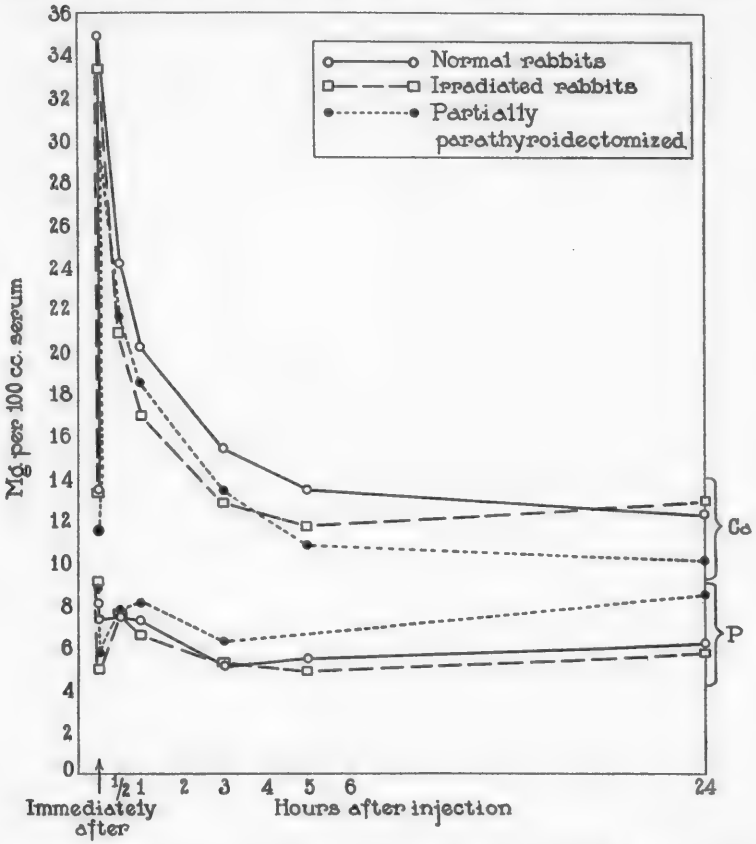

CHART 2. Experiment 2. Serum Ca after injection of $CaCl_2$ in normal, irradiated, and partially parathyroidectomized rabbits.

the inorganic phosphate levels. The initial drop is due partly to dilution of the blood serum. A reciprocal relationship is seen in the slight rise during the rapid drop in calcium.

Experiment 2.—In this experiment two normal and two irradiated rabbits were supplemented with three partially parathyroidectomized animals. Two of these had been deprived, 6 weeks before, of both external parathyroids and one-half the thyroid with its internal parathyroid gland. The other rabbit had had its external parathyroids removed 4 weeks previously. During these intervals the serum calcium of these rabbits had been gradually restored to 11.4, 12.0, and 11.2 mg. respectively, indicating a corresponding restoration of parathyroid function.

These rabbits received intravenous injections of $CaCl_2$ in sterile $\frac{M}{4}$ solution, in doses of 35 to 50 mg. of Ca per kilo of body weight. Because of the increased concentration, the total volume injected was somewhat less than in Experiment 1. Small blood samples were withdrawn from an ear vein, just before and after injection, and 30 minutes, 1, 3, 5, and 24 hours later. The results of Ca and P determinations on these specimens are shown in Chart 2.

In all of these rabbits the immediate rise of the blood Ca to a high level was followed by a rapid disappearance of the excess from the blood, so that the control levels were restored in 3 to 5 hours. No striking differences were observable between the average values for the three groups, and indeed the individual figures obtained 30 minutes and 1 hour after injection were remarkably similar throughout. Apparently the condition of the parathyroid glands had little influence on the disappearance of excess calcium from the blood stream. It is interesting to observe in the partially parathyroidectomized rabbits that the blood calcium returned to its former lower level, as Salvesen (6) and others have observed repeatedly under similar conditions.

Thus the calcium level of the blood seems to be determined by a balance between parathyroid activity and other factors independent of parathyroid control. When there is a reduction of parathyroid activity, as after partial parathyroidectomy, the calcium level drops correspondingly. But even an excess of parathyroid tissue does not maintain an excess of calcium already present in the blood, in opposition to the forces which tend to restore it to a normal level (2).

That the immediate reduction of inorganic phosphorus was due in part to the calcium content of the injection fluid is shown by the greater fall in phosphorus in Experiment 2, as compared with Experiment 1, in which a larger volume of fluid was injected. This drop was most marked in the irradiated rabbits of both series, in which an average fall from 7.83 mg. to 5.32 mg. per 100 cc. of serum occurred. The fall in phosphorus was as temporary as the cor-

responding rise in calcium and illustrates again the tendency to reciprocal reactions which several authors have already pointed out. The experiment does not indicate whether the restoration of inorganic phosphorus in the first half-hour after the calcium injection was a cause or an effect of the correspondingly rapid fall in calcium.

Phosphate Injections.

Subcutaneous injections of sterile disodium hydrogen phosphate (Na_2HPO_4) were given in $\frac{M}{2}$ solution in doses of 100, 125, or 150 mg. of P per kilo body weight.

Experiment 3.—The ten rabbits injected with 100 mg. P per kilo reacted in an essentially similar manner. Five were normal, three had been partially parathyroidectomized (two external glands and one-half the thyroid extirpated 16 to 28 days previously), and two had been irradiated daily for 23 or 26 days in increasing periods of 10 to 90 minutes at 1 meter's distance from the quartz mercury arc lamp. Blood samples withdrawn from an ear vein before the injection, and 4, 6, 12, and 24 hours afterwards were allowed to clot and the sera were analyzed for Ca (4) and inorganic P (5).

The average figures for each group closely represent the individual findings. These averages, plotted in Chart 3, show, in the first place, the somewhat lower blood calcium of the partially parathyroidectomized rabbits before the injection, and a relatively high blood phosphorus in both irradiated (3) and partially parathyroidectomized animals (7). No significant differences occurred in the reactions of the normal and the irradiated rabbits, and the partially parathyroidectomized animals differed from the others only in the fact that the lower calcium level which distinguished them was maintained throughout the period of observation. The subcutaneous injection of disodium hydrogen phosphate was followed by a prompt rise of inorganic phosphorus in the blood. The peak of the rise, as the next experiment will show, probably occurred before the 4 hour blood sample was taken. In all of these rabbits the excess phosphorus in the blood was practically eliminated within 12 hours. In fact, the most striking feature of the curves is the drop in the blood phosphorus of the irradiated and the partially parathyroidectomized rabbits well below the high levels found in their blood before the injection.

Since the blood is normally a supersaturated solution of calcium bicarbonate and calcium hydrogen phosphate (8), it is to be ex-

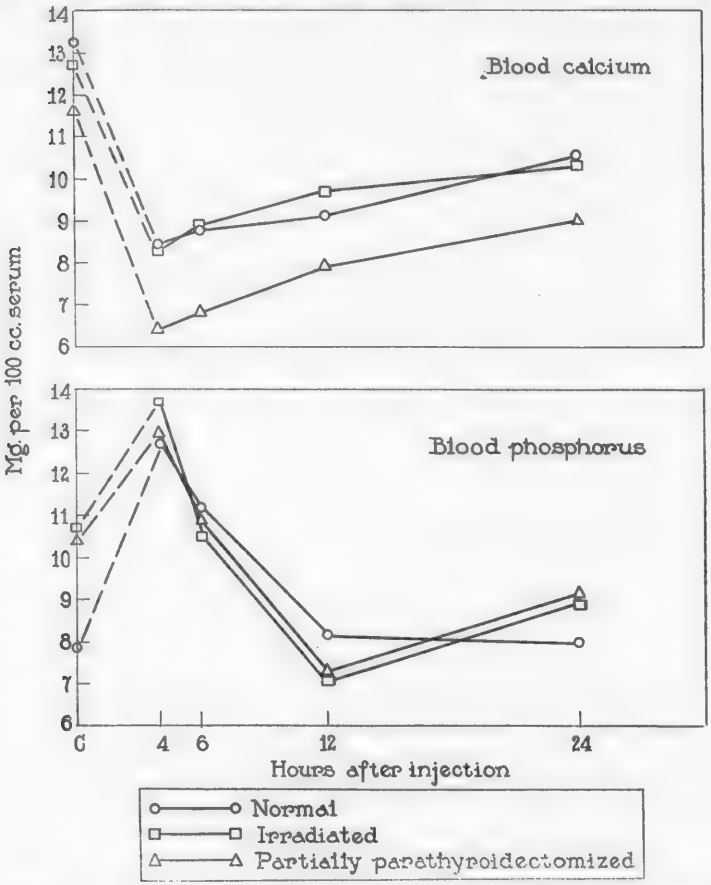

CHART 3. Experiment 3. Serum Ca and P after injection of Na_2HPO_4 in normal, irradiated, and partially parathyroidectomized rabbits.

pected that a rise in other phosphates would cause a fall in calcium. Binger (9) produced tetany in dogs by phosphate injections and found the blood calcium reduced beyond the degree attributable

to serum dilutions. Salvesen, Hastings, and McIntosh (10) have recently raised the blood phosphorus level in dogs by oral administration of mono- and disodium orthophosphates, and observed a corresponding fall in blood calcium.

In this experiment the blood calcium level fell promptly in all the rabbits, probably reaching its nadir about the 4th hour, and then rising slowly toward normal. Some scattered observations after 48 and 72 hours indicate that the normal levels were attained within that time. The lowest Ca level recorded was 6.0 mg. per 100 cc. serum, found 4 hours after the phosphate injection in a partially parathyroidectomized rabbit. This rabbit showed no outspoken tetany, but another partially parathyroidectomized animal with a Ca level of 6.4 mg. had a single convulsive seizure 6 hours after injection. All of the rabbits were restless and hypersensitive, but no other acute attacks of tetany were observed.

Although they were studied in small groups, all the rabbits that received subcutaneous injections of $\frac{M}{2}$ disodium hydrogen phosphate, representing 125 or 150 mg. of P per kilo body weight, may be considered together, for the size of the dose does not serve to distinguish their reactions, which depended rather upon the calcium and phosphorus levels attained in the blood.

Experiment 4.—Nineteen rabbits in four groups are included in this experiment: (1) four rabbits were normal, adult males; (2) four rabbits had been irradiated, three of them for 15 to 25 minutes 6 days a week for 26 to 30 days, at a distance of 50 cm. from a quartz mercury arc. The other had been exposed 10 to 90 minutes a day for 26 days at 1 meter's distance; (3) five rabbits had been irradiated with the quartz mercury arc (30 minutes daily, 18 to 21 days, at 1 meter's distance), and then partially parathyroidectomized with the removal of the two external parathyroids only. Irradiation was continued for the 13 to 16 days between the operation and the date of the phosphate injections; (4) six rabbits had not been irradiated, but underwent similar operations 13 to 16 days before the experiment. Disodium hydrogen phosphate in $\frac{M}{2}$ solution was given to all these rabbits subcutaneously in single doses, representing 125 to 150 mg. P per kilo body weight. Blood samples taken before and at intervals after the injections were analyzed for Ca and inorganic P.

In all nineteen rabbits the immediate results of these phosphate injections were a rise in blood phosphorus, a drop in the blood calcium level, and the onset of signs and behavior characteristic of

acute tetany. The reaction usually was most severe between the 4th and 8th hours, an interval in which ten rabbits died. Another

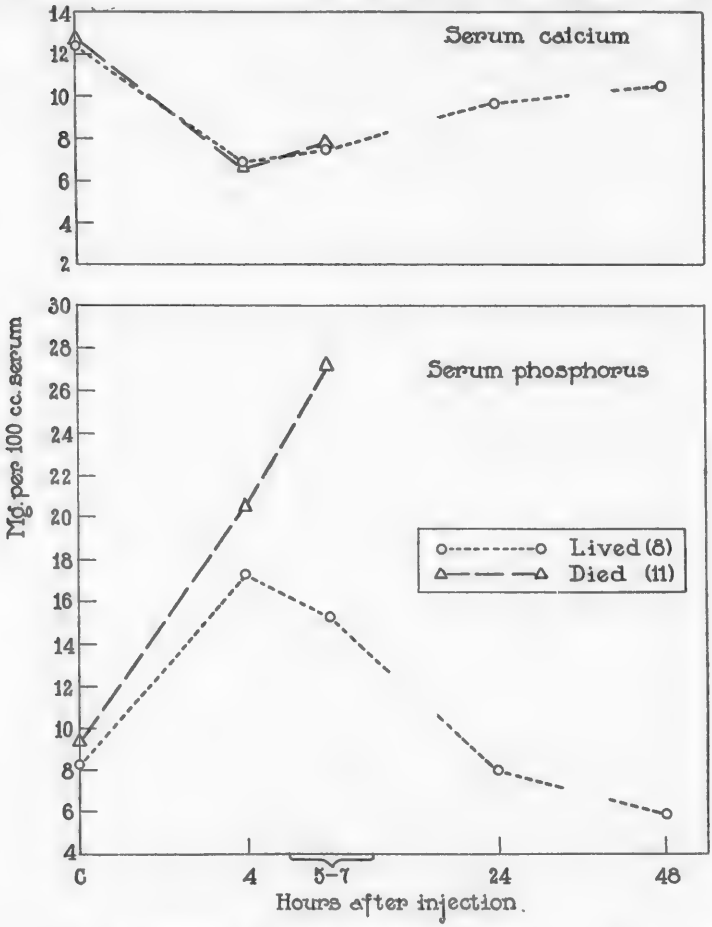

CHART 4. Experiment 4. Serum Ca and P after injection of Na_2HPO_4 showing association of P retention and death.

rabbit was found dead the following morning. The other eight, after exhibiting varying degrees of tetany, survived and returned to a state of normal irritability in 24 to 48 hours.

As already stated, the object of these experiments was to observe possible differences in the behavior of irradiated, normal, and partially parathyroidectomized rabbits to sudden changes in the calcium and phosphate balance in the blood. As in the first three experiments, so in this one a review of the rabbits' reactions shows that they cannot be distinguished according to the apparent state of the animal's parathyroid glands. Equal numbers lived and died in each group, except that among the irradiated, partially parathyroidectomized rabbits one lived and four died. Possibly these animals were more susceptible than those in the other groups. On the whole, the experiment failed to show differences in reaction which might be referred to differences in parathyroid function due to irradiation or partial parathyroidectomy.

But an analysis of the figures shows that a significant division may be made between all those that lived and those that died, on the basis of their blood calcium and phosphate levels at the onset of tetany (Chart 4). We may therefore turn our attention to this aspect of the situation and analyze the experimental findings from this point of view. The rabbits, regardless of the apparent state of their parathyroid glands, have been grouped accordingly in Table I.

In the first place, the control figures for both groups show less variation in the calcium levels than in those for phosphorus, before injection. Those that lived are not different in blood calcium from those that died, either individually or on the average. But in the group of eight survivors there are only three with a blood phosphorus, before injection, above 7 mg. per 100 cc. of serum, while among those that died nine of the eleven had high control levels, indicating, possibly, an inherent tendency to phosphorus retention.

As is shown by the available 2 hour determinations, the first peak of phosphorus in the blood probably occurred in most instances before the 4 hour specimen was taken. Of the nine rabbits examined at 2 hours, four survived and five died. Those that survived showed at the 4 hour period a considerable drop in blood phosphorus to a level which was maintained or further decreased at 5 to 7 hours. Some rabbits that died, on the other hand, had even higher levels at 4 hours than at 2, and the high concentrations of phosphorus in their blood at 5 to 7 hours are in marked contrast to the correspond-

TABLE I.

Lived

Rabbit No.	Condition	Serum Ca mg. per 100 cc.						Serum P mg. per 100 cc.					
		Control	Hrs. after injection					Control	Hrs. after injection				
			2	4	5–7	24	48		2	4	5–7	24	48
22	N	11.0	6.3	5.7	7.2	8.8	11.2	5.6	19.7	15.6	14.6	7.5	6.5
24	N	12.3	7.2	6.5	6.3	7.8	11.0	5.2	18.1	16.8	17.0	9.0	5.8
26	I	14.6	—	7.4	7.8	10.2	11.0	13.0	—	17.1	17.4	9.6	—
29	I	12.8	8.6	7.9	7.9	12.4	—	6.6	25.7	17.1	11.5	5.0	—
35	P P	12.8	—	7.4	—	6.4	5.8	15.3	—	20.6	—	18.0	7.4
34	P P	13.7	—	6.7	—	11.8	12.7	5.7	—	12.8	—	4.3	5.2
31	P P	12.9	—	7.3	—	10.8	11.4	5.8	—	15.1	—	4.7	4.9
37	I P P	10.8	7.5	6.7	8.1	9.2	—	9.5	29.2	23.8	15.7	6.0	—
Average.....		12.6		7.0	7.5	9.7	11.0	8.3		17.4	15.2	8.0	6.0
						Died						Death at	
25	N	13.3	7.5	5.5	6.7			10.3	24.2	25.6	22.1	5 hrs., 18 min.	
23	N	11.9	7.5	6.7	7.9			9.9	17.2	19.2	22.5	6.05	
27	I	13.1	7.3	6.3	6.5			12.0	27.7	21.7	21.7	7–8 hrs.	
28	I	12.0	8.6	8.2	8.1			6.2	24.0	18.4	24.8	First night	
30	P P	13.0	—	7.1	7.3			6.8	—	14.8	33.0	7.05	
32	P P	12.9	—	6.5	—			7.6	—	25.9	—	3.54	
33	P P	13.5	8.8	7.5	8.4			15.0	24.2	23.1	38.7	6.28	
40	I P P	12.8	—	6.5	7.3			7.6	—	21.8	28.4	7.18	
39	I P P	11.8	—	6.7	7.7			8.8	—	21.4	30.0	6.53	
36	I P P	12.9	—	7.5	—			8.2	—	15.4	—	5.27	
38	I P P	13.1	—	6.3	9.6			8.8	—	19.2	24.0	5.30	
Average.....		12.8		6.8	7.7			9.2		20.6	27.2		

N = Normal.
I = Irradiated.
P P = Partially parathyroidectomized.
I P P = Irradiated and partially parathyroidectomized.

ing figures for the survivors. The fate of these rabbits, therefore, depended not on the primary blood phosphorus level attained shortly after injection, but on the elimination or retention of this excess of

phosphorus in the course of the next few hours. Every rabbit (but one ?) that died had a blood phosphorus level above 21 mg. per 100 cc. serum, 4 to 7 hours after injection. Every rabbit that survived certainly or presumably had a blood phosphorus level below 21 mg. at the same period.

With reference to the occurrence of acute tetany, it is essential that the corresponding figures for blood calcium be taken into consideration. The 2 hour specimens showed a marked drop in blood calcium from the control figures, and this drop was even greater after 4 hours and often reached levels commonly associated with the signs of acute tetany, which all these animals displayed. But it is manifestly significant that differences in the blood calcium level do not distinguish the surviving rabbits from those that succumbed. In the rabbits that died, although the initial drop in blood calcium coincided with the great initial rise in blood phosphorus, the secondary rise in phosphorus that preceded death was in no observed instance accompanied by a further drop in calcium (11), so that just before death occurred the blood calcium in these rabbits was usually somewhat higher than it had been an hour or more previously. Low calcium alone cannot be held responsible for the death of these rabbits.

In the first paper of this series we developed the hypothesis that absolute figures for serum calcium or serum phosphorus are not so significant in experimental tetany as is the ratio between the two elements in the blood. Howland and Kramer (8, 12) have pointed out that in normal children this ratio is approximately 2:1, a proportion most favorable to bone formation, and that in rickets changes in the ratio occur, which may explain, in part, the failure of calcium phosphate deposition. Normally in rabbits also the ratio Ca/P is greater than 1. Among 103 rabbits the average ratio was 1.9:1. In these phosphate-injected animals, however, especially in the instances of phosphate retention in the blood, the ratio fell to a small fraction of its former value. These low ratios are obviously of more significance than are the individual calcium levels alone in interpreting the occurrence of severe and lethal tetany.

In considering the significance of these relative changes in Ca and P concentration, the fact that considerable amounts of the mono-

valent sodium ion were also injected probably must be taken into account. One well established theory of hyperirritability and tetany is based on disturbances in balance between monovalent and divalent cations and anions in the blood. In a very sane and illuminating review and discussion of this question Hastings and Murray (11) weigh the relative importance of monovalent cations and divalent anions as stimulants to nerves. When disodium hydrogen phosphate is injected, a "double dose," so to speak, of nerve irritants is given, and it is hardly profitable to speculate as to which is the chief offender.

It is not our intention on the basis of these few experiments and those reported in the first paper of this series to undertake a critical discussion of current theories of the cause, or more probably causes, of experimental tetany. This vexed and complicated question is not germane to the original object of this investigation. That specific object was attained in the discovery that no significant differences were observable in the reactions of irradiated, normal, or partially parathyroidectomized rabbits to injections of calcium chloride or of disodium hydrogen phosphate.

SUMMARY.

No significant differences were found in the reactions of irradiated, normal, or partially parathyroidectomized rabbits to injections of calcium chloride or disodium hydrogen phosphate.

Intravenous injections of calcium chloride caused only a transient rise in blood calcium, which returned to its former level within a few hours. The upper level of blood calcium concentration is independent of parathyroid control.

Subcutaneous injections of disodium hydrogen phosphate caused, in all three groups of rabbits, a similar depression of the blood calcium level. After large doses of the phosphate solution, all of the rabbits showed signs of acute tetany in which many of them died. Death or survival was not determined, apparently, by the absolute depression of blood calcium. The rabbits that died were those, in all three groups, in which a phosphorus retention carried the initial rise in serum phosphorus to even higher levels in the hours preceding death, and so changed the normal calcium-phosphorus ratio to a small fraction of its former value.

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EXPERIMENTAL OBSERVATIONS ON IRRADIATED, NORMAL, AND PARTIALLY PARATHYROIDECTOMIZED RABBITS.

III. The Effects of Inanition.

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In order to test the functional significance of the parathyroid hypertrophy due to ultra-violet irradiation of normal rabbits (1) we have subjected irradiated rabbits to partial parathyroidectomy (2) and have compared the effects of injections of calcium chloride and of disodium hydrogen phosphate in irradiated, normal, and partially parathyroidectomized animals (3).

A third method of questioning the significance of this parathyroid hypertrophy was to observe the effects of complete inanition in irradiated, normal, and partially parathyroidectomized[1] rabbits to determine possible differences in the fasting metabolism of calcium and inorganic phosphorus with which the parathyroid glands, directly or indirectly, are so essentially concerned.

Much of the recent work on the physiological and therapeutic effects of ultra-violet light has been devoted to studies on various aspects of metabolic activity, especially with diets which are vitamine-poor, or incomplete in other respects. A fasting experiment with irradiated rabbits also promised opportunities for observation of certain effects of ultra-violet light on metabolism and further information in regard to the action of radiant energy on the endocrine glands.

Accordingly two experiments were undertaken, in which different groups of irradiated, normal, and partially parathyroidectomized rabbits were subjected to fasts of 5 and 11 days, respectively. The

[1] All operations for removal of parathyroids were performed under full ether anesthesia.

5 day period proved too short to yield results of definite significance, and was complicated by the ingestion of unknown amounts of sawdust and feces from the individual cages, so it will not be reported in detail. In outline the experiment ran as follows:

Experiment 1.—This experiment was started with fifteen adult male rabbits in three groups. Five served as normal controls. Five had been irradiated for 44 days with a quartz mercury arc at 1 meter's distance with daily exposures of 10 minutes to 2 hours. The 2 hour exposure was continued during the fasting period. The other five rabbits had been partially parathyroidectomized with the removal of both external parathyroids and half the thyroid gland with its internal parathyroid 50 to 34 days previously. Except in one instance the remaining internal parathyroid gland in these rabbits was maintaining a calcium level of 12.0 to 13.4 mg. per 100 cc. serum. The exception, a rabbit with a control blood calcium of 9.3 mg. and inorganic phosphorus of 8.4 mg. per 100 cc. serum, died on the 5th day of the fast with a blood Ca of 5.7 mg. and P of 12.0 mg. per 100 cc. serum. The rabbits were kept in their regular sawdust-strewn individual cages and given 100 cc. of distilled water by stomach tube daily. Blood samples were taken from the ear before the fast, on the 2nd and 5th days, and 2 to 4 days after feeding had been resumed. The serum specimens were analyzed for Ca (4) and inorganic P (5).

One of the irradiated rabbits was killed by accident on the 5th day of the fast. Autopsy revealed a certain amount of hair, fecal matter, and sawdust in his stomach. Presumably the other rabbits also had ingested indefinite amounts of sawdust and feces, so that the results of this experiment are not open to a strict interpretation. But the average figures for each group show certain tendencies in calcium and phosphorus metabolism that lend emphasis to the results of the second experiment.

Eliminating from the averages for each group the figures obtained from the two rabbits that died and from two others, one normal and one partially parathyroidectomized, for which our records are incomplete, the results of this experiment are shown graphically in Chart 1.

A primary effect of fasting may be seen in the rapid fall in blood calcium that occurred in all of the rabbits. As might be expected, the total loss of calcium by the partially parathyroidectomized rabbits was somewhat greater than that in the other groups. In the first few days after feeding was resumed the blood calcium was restored almost as rapidly as it had been lost during inanition. The more rapid rate of calcium accumulation in the irradiated and partially parathyroidec-

tomized rabbits, as compared with the normals, may be due to a more active state of their parathyroid tissue.

A reciprocal relationship between calcium and inorganic phosphorus may be illustrated by the inverted curves for phosphorus in the irradiated and normal rabbits. Inorganic phosphorus in the blood serum was above normal in these animals during the fasting period and dropped again when the calcium supply was restored. The partially parathyroidectomized rabbits, on the other hand, lost both calcium and phosphorus from the blood;—except in the instance noted above (and not included in the average figures), in which a drop in calcium

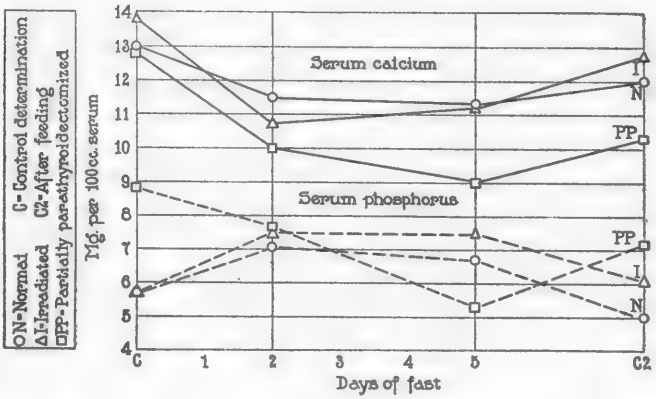

CHART 1. Experiment 1. Serum Ca and P during inanition in normal, irradiated, and partially parathyroidectomized rabbits.

to 5.7 mg. and a rise in phosphorus to 12.0 mg. per 100 cc. serum preceded the rabbit's death.

During this short fasting period none of the rabbits suffered an undue loss in body weight and the average losses for all three groups was less than 10 per cent.

After the final blood samples were obtained, the normal and the irradiated rabbits were killed and autopsied to determine the relative size of their external parathyroid glands. The two external glands of the irradiated rabbits averaged 8.1 mg. per kilo net body weight; those of the normal rabbits averaged 5.7 mg.

In the second experiment the individual metal cages were fitted with raised bottoms of wire mesh through which the urine and feces could fall freely, so that the rabbits ingested nothing but distilled water during the fast.

Experiment 2.—This experiment was performed with fifteen adult male rabbits in three groups. Five rabbits served as normal controls. Five rabbits with ears shaved and backs clipped had been irradiated 2 to 22 minutes daily (except Sundays) for a period of 20 days at 50 cm. distance from a 67 volt, 5.5 ampere quartz mercury arc. During the fast, daily exposures of 23 to 30 minutes were given. The other five rabbits had been deprived of their two external parathyroid glands 10 days previously. The three groups of rabbits were subjected to an 11 day fasting period.

The rabbits were each given 100 cc. of distilled water daily by stomach tube, and were bled from an ear vein for serum samples before the fast, on the 4th, 7th, and 11th days of inanition, and, after feeding had been resumed, on the 12th, 15th, and 18th days of the experiment. The serum samples were analyzed for calcium and inorganic phosphorus. The rabbits were weighed on alternate days.

Two rabbits, one irradiated and one partially parathyroidectomized, died on the 9th day of the fast, and since the figures for Ca and P obtained from them have been excluded from the averages in Chart 2 they must be dealt with separately. The irradiated rabbit started the fast with a serum Ca of 12.7 mg. and P of 8.8 mg. per 100 cc. serum. On the 7th day its serum Ca and P were 8.7 mg. and 10.7 mg. respectively. In 9 days of inanition this rabbit lost 52 per cent of its original body weight and its death is ascribed to causes associated with this great loss, rather than to tetany. The partially parathyroidectomized rabbit had not restored its blood calcium as promptly as is usual after such an operation (2) and started the fast with a serum calcium of 8.9 mg. and phosphorus of 8.8 mg. per 100 cc. serum. On the 4th day serum Ca was 7.2 mg. and on the 7th day 4.9 mg. The corresponding figures for P were 11.3 mg. and 12.7 mg. respectively. Although the death of this animal was not observed, such figures are typical of acute tetany.

Three of the four irradiated rabbits that survived the actual period of inanition died within the next 5 days. The normal and partially parathyroidectomized rabbits were allowed to survive in order to study the period of their recovery. 2 months later a second group of five normal rabbits was subjected to a similar fast and killed at its conclusion to provide a comparison at autopsy with the endocrine glands of the irradiated rabbits. In Chart 2 the figures for Ca and P in the serum of this second group of normal fasting rabbits have been averaged in the curves marked N2.

The figures for calcium and inorganic phosphorus (in mg. per 100 cc.

serum) in the blood of the rabbits which survived the actual period of inanition have been averaged for each group in Chart 2. With few exceptions these averages fairly represent the individual calcium findings. A bizarre and unexplained figure of 15.1 mg. for serum Ca in one of the irradiated rabbits, and a single high Ca of 12.45 mg. in one of the partially parathyroidectomized rabbits on the 4th day of the fast disturb the downward trend of the calcium averages, and have

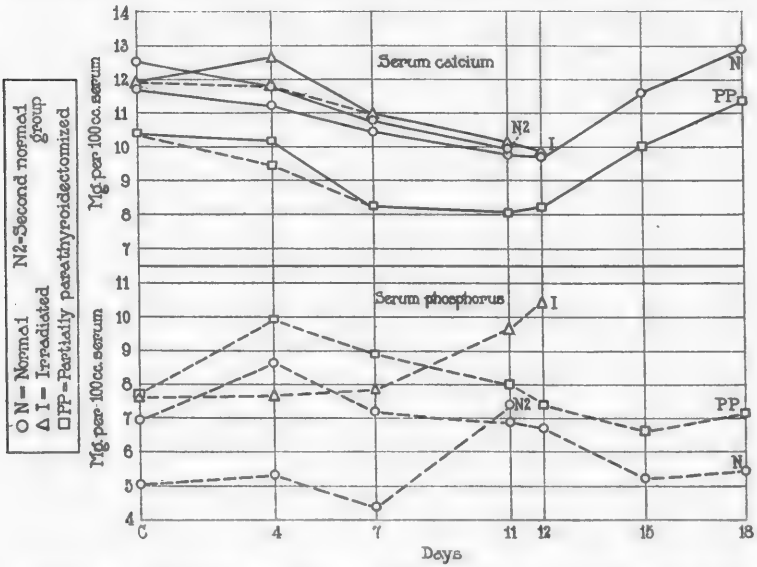

CHART 2. Experiment 2. Serum Ca and P during inanition and after feeding in normal, irradiated, and partially parathyroidectomized rabbits.

been omitted in the alternate averages for that day, also shown in the chart. The corresponding figures for phosphorus in these two animals were not different from those of other rabbits in the same groups.

As in the first experiment, inanition caused a progressive loss in serum calcium in all three groups of rabbits. A similar loss in serum phosphorus did not occur. We shall not attempt to interpret this tendency to phosphorus retention. Wide variations occurred in the figures for the individual rabbits and the averages merely cancel these

variations, so that no group stands out as manifestly different from the others in regard to phosphorus metabolism during the fast. Yet the fact that inanition does not regularly cause a progressive drop in the inorganic phosphorus of the blood serum is shown by the maintenance of the phosphorus levels in six of the seven groups in Experiments 1 and 2.

In regard to serum calcium and inorganic phosphorus metabolism, then, only one difference appears which can be interpreted as due directly to differences in parathyroid function among the four groups of rabbits. The lower level of serum calcium in the partially parathyroidectomized rabbits was maintained throughout both experiments. But the trend of the calcium level in this group was not different from that in the others.

Mention has been made of the fact that one irradiated rabbit died on the 9th day of the fast and that three of the four survivors died within 5 days after feeding was resumed. With the single exception of the partially parathyroidectomized rabbit that died on the 9th day, apparently of tetany, no similar fatalities occurred among the other groups. In general the 11 day period of inanition was well borne by all but the irradiated rabbits. The cause of the deaths in this group must be sought among the direct or indirect effects of the exposure to ultra-violet light. The dosage used, 5 to 30 minutes at 50 cm. distance, is not excessive and had been well borne by normal rabbits. Although the serum phosphorus was high in three of the four victims, serum calcium was not unduly low in the last determination made before death.

Aside from its hypertrophic effect on the parathyroid glands ultra-violet light has two possible effects on the animal which must be taken into account in this experiment. One is on general metabolism. The other is adrenal injury.

A comparison of the relative loss of weight by the three groups of irradiated, normal, and partially parathyroidectomized rabbits shows that from the first the irradiated animals lost weight more rapidly during inanition than did the other groups and toward the end of the fasting period this loss was exaggerated. Even during the first 4 or 5 days after feeding was resumed the weight of most of these rabbits remained stationary or declined further, and it was during this period

that three of the four surviving irradiated rabbits died, each at the nadir of his body weight curve. While the five normal rabbits lost an average of 31 per cent in body weight, and the four surviving partially parathyroidectomized rabbits lost 29 per cent, the five irradiated rabbits lost 52, 41, 39, 48, and 38 per cent of their control weights, respectively, and the first four of these rabbits died on the 9th, 12th, 13th, and 16th day of the experiment.

If loss in body weight be taken as an index of the metabolic rate in these rabbits, the effect of exposure to the quartz mercury lamp is at once apparent. In judging the effects of the exposure two factors have to be taken into account. One is the direct action of visible and ultra-violet light. The other is the handling and the movements of the rabbits due to the daily exposure. All of the rabbits were brought to the laboratory daily for water by stomach tube. In addition, the irradiated group was placed for about half an hour in a galvanized iron tub in which movement was not so restricted. In general they remained quiet, and we do not believe that this extra handling of the irradiated rabbits can account for their excessive losses of weight and for their deaths.

A review of the literature on the effects of visible and ultra-violet light on basal metabolism indicates that experimental conditions and methods have varied so widely that few useful comparisons can be made.

Thus Kestner, Peemöller, and Plaut (6), using a quartz mercury arc lamp in a study with the Benedict apparatus, produced a transient rise in metabolism in children and adults, but Hill and Campbell (7) call attention to the effects of cool air during exposure and consider that the rise in the metabolic rate caused by heliotherapy *per se* is relatively insignificant. Fries (8) found no appreciable difference in basal metabolism of children during treatment with a quartz mercury lamp compared with intervals without treatment, but thinks that the reaction of the individual must be taken into consideration. Recently Campbell (9) has reported a carefully planned and executed series of experiments with rats and mice in a specially adapted metabolism chamber. The oxygen consumption and the carbon dioxide output of four men were tested also, and the author concludes that irradiation with the whole or part of the spectrum of a quartz mercury vapor lamp has no effect on the metabolism of healthy men, rats, or mice. This is a sweeping statement in view of the fact that only the immediate or current effects of the radiation were investigated.

Northrop (10) in a precise experiment with aseptic cultures of *Drosophila*, grown for 200 generations in the dark, found that the light of a tungsten filament

lamp, through water, had marked effects on growth and duration of life. With intensities greater than 1000 meter candles the duration of the imago stage was rapidly shortened and the duration of life of the imago could be predicted by assuming that the light ages the flies independently of the normal rate. The light effect was proportional to its intensity.

In the absence of observations on basal metabolism in our groups of fasting rabbits, we can only call attention to the excessive loss of weight in the irradiated rabbits, and suggest its possible relation to an increased metabolic rate resulting in the more rapid consumption of the animal's own tissues. A definite effect of fasting and the exposures to radiant energy was found at autopsy in the irradiated victims of the fast.

From time to time we have observed gross hemorrhages in the suprarenal glands of irradiated rabbits (1). The point of origin of these hemorrhages has usually been the medullary tissues, but they have often extended into the cortex and even to the capsule of the affected gland. The frequency of such a lesion after irradiation, even when only moderate erythema doses have been given, has raised in our minds the question of a specific secondary action of ultra-violet energy on these essential organs. The effects of such an injury might be reflected clinically in blood pressure changes, which were not studied in these fasting rabbits. Under other conditions, light, especially ultra-violet light, has often been observed to lower blood pressure (11) and the signs of collapse that sometimes follow dangerous degrees of sunburn are in certain respects similar to the signs of suprarenal insufficiency (12).

According to Jackson (13), and other authors whom he quotes, inanition and various types of malnutrition often cause suprarenal congestion and hemorrhage. In fasting the suprarenals do not show a proportionate loss in weight, sometimes being relatively twice as large as is normal. Congestion, and sometimes hemorrhage, is characteristic in both cortex and medulla and may account for the increased weight in spite of atrophy of the parenchyma. Congestion and hemorrhage, gross or capillary, have been reported also in athrepsia, scurvy, and pellagra, so it appears that the suprarenal glands often suffer heavily in states of malnutrition.

With two causes of suprarenal damage operating in conjunction, it

is hardly surprising that three of the four irradiated rabbits examined at autopsy showed scars and medullary hemorrhage in one or both of these essential glands. One gland of the fourth rabbit was lost, and its condition cannot be reported. The other gland appeared to be normal. None of the lesions were so recent as to suggest that they were agonal, or the immediate cause of death, but suprarenal insufficiency may have contributed to the high mortality among the irradiated rabbits.

TABLE I.

Glands	Gland weights (in mg. per kg. net body weight)				Change in gland/body weight ratio, effect of					
	Normal		Irradiated		Irradiation		Fasting		Both	Fasting
					I	II	III	IV	V	VI
	Controls	Fasted	Controls	Fasted	IC/NC	IF/NF	NF/NC	IF/IC	IF/NC	Jackson
Pineal	6.7	11.3	10.2	14.1	+	+	+	+	+	±
Hypophysis	14.8	18.2	16.9	29.6	+	+	+	+	+	+
External parathyroids	6.1	5.5	9.2	7.0	+	+	−	−	+	−
Thyroid	112.8	116.4	92.2	156.2	−	+	±	+	+	+
Thymus	1151.	390.	1111.	402.	±	±	−	−	−	−
Suprarenals	195.0	279.7	212.5	374.6	+	+	+	+	+	+
Testicles	2828.	3122.	2670.	3066.	±	±	+	+	+	−

N = Normal. I = Irradiated. C = Control. F = Fasted.
+ = Gland spared. Weight of gland increased relative to body weight.
− = Gland consumed. Weight of gland decreased relative to body weight.

At autopsy other endocrine glands of the fasted, irradiated rabbits, and of the fasted, normal rabbits of Group N2, were weighed and their weights compared with figures obtained for other irradiated and normal rabbits at a corresponding season of the year. These figures and a crude analysis of them have been collected in Table I, which may require some explanation.

The figures given represent gland weights, in mg. per kilo of net body weight, after the removal of the gastrointestinal tract and its contents, and the expression of bladder urine. For the normal control figures we are indebted to Dr. Louise Pearce. In the second half

of the table an attempt has been made to indicate whether fasting spares these essential glands, or exhausts and consumes them more rapidly than other body tissues. The effect of irradiation alone is seen in the gland weight ratios of irradiated and non-irradiated rabbits in both the control and the fasting groups. An idea of the effect of fasting alone is gained by comparing fasted with control rabbits in the normal and irradiated groups. A summation of the effects of irradiation and fasting is found in a comparison of the figures for the irradiated fasted rabbits with the normal controls. Considering the small number of fasted rabbits available for study, the results of the analysis are in good accord. The effects of irradiation alone, Columns I and II, check in most instances both for normal and for fasted rabbits and in the control, irradiated, rabbits the same effects of irradiation are seen as have already been reported (1): pineal, hypophysis, external parathyroid, and suprarenal glands are relatively heavier than normal, the thyroid gland has lost weight, and the thymus and testicles show little change. Similarly the effects of inanition on these endocrine glands may be seen in Columns III and IV. Pineal, hypophysis, thyroid, suprarenals, and testicles are spared in both groups of rabbits. The external parathyroids, and especially the thymus are consumed relatively faster than the body as a whole. Finally, in the single instance in which the effects of fasting and of irradiation on gland weight seem to be opposite, *i.e.* the effect on the external parathyroid glands, the light treatment has more than offset the effects of fasting, and the parathyroids in the irradiated fasted rabbits are larger than is normal. But in spite of the relatively large size of the external parathyroid glands in the irradiated rabbits, their curves for serum calcium during the fasting period in both experiments are essentially similar to those for the normal groups (see Charts 1 and 2).

In most instances these tendencies to the protection or exhaustion of the endocrine tissues in our fasted rabbits correspond with the effects of inanition in man and other animals, reviewed and summarized by Jackson (13). His general conclusions in regard to each gland are indicated in Column VI in the table, and may be noted briefly as follows:

Pineal Gland.—No appreciable change relative to body weight. No conclusions drawn.

Hypophysis.—Loss of weight relatively less than that of the body as a whole.

Parathyroid Glands.—Usually found to be atrophic. In rats Jackson found them reduced in weight nearly in proportion to the body loss. They tend to be relatively decreased in weight in adults, with a variable degree of atrophy and an increase in fibrous stroma.

Thyroid Gland.—The loss of weight is variable, usually relatively less than that of the body as a whole.

Thymus.—The extreme loss of weight of this gland is rivaled only by that of adipose tissue. The loss is relatively far greater than that of the body and usually reaches 75 per cent before death from acute inanition.

Suprarenals.—These glands are usually increased in weight but part of this increase may be due to hemorrhage.

Testicles.—The loss in weight is relatively greater than that of the body as a whole.

Thus the only result of our observations that is at variance with Jackson's conclusions is in the loss of weight of the testicles relative to general body weight loss. But these glands normally vary widely in absolute and relative weight, and we lay no stress upon the aberrant results of our single experiment.

SUMMARY AND CONCLUSIONS.

The experiments described in the three papers of this series were undertaken to determine if possible the significance of the parathyroid hypertrophy that has repeatedly been found in rabbits after exposure to ultra-violet light. The fact that the hypertrophy was not accompanied by a corresponding increase in blood calcium led us to infer that the upper level of blood calcium concentration is governed by factors independent of parathyroid control, and that the gland hypertrophy under the influence of ultra-violet light might represent a potential functional capacity, or increased factor of safety, to protect the calcium level under conditions of stress or emergency. Partial parathyroidectomy in irradiated rabbits, compared with the same operation in normal controls, resulted in a similar immediate drop in blood calcium; but a more rapid restoration of the calcium level in the irradiated rabbits indicated a more prompt and active response to the emergency on the part of the remaining glands. In the second group

of experiments no significant differences were found in the reactions of irradiated, normal, or partially parathyroidectomized rabbits to injections of calcium chloride or of disodium hydrogen phosphate. All three groups of rabbits rapidly eliminated the excess calcium from the blood. Their reactions to large doses of sodium phosphate, given subcutaneously, appeared to be related to phosphate elimination or retention in the individual animals. Those in each group which had phosphate retention in the blood died in acute tetany.

So also in the fasting experiments, reported in this paper, no distinctions could be drawn between normal, irradiated, and partially parathyroidectomized rabbits on the basis of their calcium and phosphorus metabolism. Blood calcium was reduced in a similar manner in all three groups of rabbits, and wide individual variations in serum phosphorus levels preclude significant deductions.

But the irradiated rabbits suffered disproportionate losses of weight during the fasting period and four of five of them died during the course of the experiment. The deaths of these rabbits are attributed to this more rapid consumption of their own tissues and possibly also to suprarenal injury caused both by fasting and by exposure to ultraviolet light.

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STUDIES ON BACTERIAL ENZYMES.

I. Meningococcus Maltase.

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INTRODUCTION.

The fermentation of maltose is one of the most important characters employed in the differentiation of meningococci from some of the saprophytic cocci found in the respiratory tract. It is of importance, therefore, to determine whether maltose hydrolysis can be effected by enzymes liberated from the bacterial cells. In the present paper this question has been answered by an investigation of the maltose-splitting capacity of sterile filtered solutions of the intracellular substances of meningococci.

Measurement of the action of maltase involves an estimation of glucose in the presence of maltose. Since both are reducing sugars, glucose in such mixtures cannot be estimated by the usual, simple or direct copper reduction methods. Polarimetric determination of glucose requires special apparatus not always at hand in the bacteriological laboratory. Barfoed's method is applicable to the detection of glucose in the presence of maltose, but requires rigid controls especially when the amount of maltose greatly exceeds the glucose. Preliminary tests by this method on mixtures of the bacterial enzyme and maltose did not prove satisfactory. Precise readings were difficult to make because of the voluminous precipitation of the phosphate in the test solutions by the acetic acid in Barfoed's reagent.

Since only qualitative estimations were desired, a biological method was employed, which is based upon the acid fermentation of glucose by bacteria which have little or no action upon maltose.

EXPERIMENTAL.

Methods.

Preparation of Meningococcus Enzyme Solutions.—The enzyme solution was prepared from a strain of normal meningococci (Type II Gordon) furnished by Dr. Elliott S. Robinson of the Massachusetts Antitoxin and Vaccine Laboratory.

Meat infusion agar enriched by the addition of Avery's (1) yeast extract was inoculated heavily with young meningococci suspended in infusion broth. Luxuriant growth was always obtained within 12 to 18 hours; the meningococcus cells were then scraped off the surface of the agar and suspended in sterile 0.1 M phosphate (pH 7.5). Since the medium in which the bacteria were suspended included the broth introduced with the original inoculum, the final solution in which the bacteria were extracted contained about an equal volume of infusion broth and phosphate solution. The meningococcus suspension was placed in long narrow Pyrex tubes, sealed with vaseline, and repeatedly frozen and thawed as in the preparation of sterile pneumococcus extracts (2).

The suspension of disintegrated bacteria was centrifuged at high speed for 1 hour; the supernatant was then pipetted off from the bacterial residue. To eliminate any possibility of the presence of intact meningococcus cells in the enzyme solutions, the supernatant was finally filtered through a Berkefeld candle.

Preparation of Maltose Solutions.—To avoid the hydrolysis which frequently accompanies the sterilization of dilute solutions of maltose, the sugar was heated in concentrated solutions. 30 per cent solutions of maltose were boiled for 15 minutes and then added aseptically to sterile 0.1 M phosphate (pH 6.9) in amount sufficient to give a final concentration of 3.0 per cent maltose. The final maltose solution was distributed into sterile Pyrex tubes.

The Detection of Glucose by Bacterial Acid Fermentation.—The procedure used to detect glucose by bacterial acid fermentation consisted in the following steps: (1) the preparation of hydrolysis mixtures (maltose plus meningococcus enzymes); (2) the incubation of the hydrolysis mixture to allow time for the action of the enzyme upon maltose; (3) the addition of certain glucose-fermenting bacteria to samples of the hydrolysis mixtures and measurement of the acid produced by the bacterial fermentation of glucose.

1. "Hydrolysis Mixtures."—The hydrolysis mixtures consisted of maltose solution plus unheated, active meningococcus enzyme solution and control mixtures of maltose solution plus heat-inactivated enzyme solution. These mixtures were incubated for 48 or 72 hours to allow time for the enzyme action.

2. "Fermentation Mixtures." (*a*) *Bacterial Fermenting Agents Employed.*—The bacteria used to detect the presence of glucose consisted of a strain of Shiga dysentery bacillus and an atypical strain of colon bacillus. (The strain of Shiga dysentery bacillus possessed the usual fermentation characteristics of these bacteria. The atypical strain of colon bacillus was isolated from stools by Mr. William L. Fleming. This strain, at least when first isolated, fails to ferment maltose al-

though actively attacking both glucose and lactose with prompt acid and gas formation, which is a comparatively rare combination of fermenting properties among the known intestinal bacteria.) Ideal fermenting agents for the detection of glucose in mixtures of maltose and glucose should possess the property of fermenting the hexose with acid production but should be without action upon maltose. Although it was found that concentrated suspensions of the strains used in our experiments do form small amounts of acid in maltose solutions, it simplifies the description of the method to define them as "non-maltose-fermenting bacteria." Any error from the slight fermentation of maltose by the "non-maltose-fermenting" bacteria was eliminated by the rigid controls described below.

The bacteria actually used as fermenting agents were taken from young agar cultures and were suspended in equal parts of meat extract broth and salt solution.

(*b*) *Preparation of the "Fermentation Mixtures."*—The "fermentation mixtures" were prepared as follows: An aliquot of each hydrolysis mixture, usually 0.5 cc. or 0.7 cc., was put into a series of small sterile Pyrex test-tubes. Then an equal volume of a suspension of the bacterial fermenting agent was added to each of the samples under test for the presence of glucose.

Although the reaction of the hydrolysis mixture was pH 6.9, it was our practice to adjust the initial reaction of the fermentation mixture to approximately pH 7.5. Not only is this pH nearer the optimum for acid fermentation, but with the initial reaction at pH 7.5 the production of small amounts of acid causes larger changes in pH than at pH 6.9 where the "buffer value" of the phosphate is at its maximum. A further advantage of having the more alkaline initial reaction is that smaller decreases in pH can be accurately estimated between pH 7.5 and pH 6.9 by the use of phenol red (3), than can be detected with brom-cresol purple in a zone slightly below pH 6.9. The reaction of the bacterial suspensions, therefore, was adjusted to approximately pH 8.5 which trial tests showed was sufficiently alkaline to yield a pH of 7.5 in a mixture of equal parts of the bacterial suspension and pH 6.9 (0.1 M) phosphate.

(*c*) *Incubation of "Fermentation Mixture" for Acid Fermentation of the Glucose.*—The "fermentation mixtures" (test solution plus glucose-fermenting bacteria) were shaken and then incubated in the water bath at 38°C. for 45 minutes. The factors to be considered in choice of the length of the incubation period will be considered below (Fig. 1).

(*d*) *Estimation of Acid Produced in the "Fermentation Mixture."*—0.5 cc. portions of properly diluted solutions of phenol red or of brom-cresol purple were placed in a series of small selected tubes of equal bore. Colorimetric standards were prepared by adding 0.5 cc. of buffer mixture to the appropriate indicators. 0.5 cc. portions of the centrifuged supernatants of the fermentation mixtures were then added to tubes containing the indicators. These supernatants were themselves practically water-clear and were diluted with an equal volume of the indicator solution so that accurate readings could be obtained by direct comparison with the buffer standards.

Controls on Acid Production in the "Fermentation Mixtures."—The following controls were used to limit the changes in reaction of the fermentation mixture to

the acid formed by the fermentation of glucose previously hydrolyzed by the active meningococcus enzymes.

1. Errors due to any hydrolysis of the maltose by some other agency than the active meningococcus enzyme were eliminated by a control series of heat-inactivated enzyme plus maltose. This series was incubated under the same conditions as the corresponding mixtures of active enzyme plus maltose.

2. In some of the experiments, slight differences existed in the initial pH of the different mixtures of enzyme plus maltose. This was controlled by preparing a duplicate series of "fermentation mixtures" in which heat-killed bacteria were used instead of active bacteria.

Slow Fermentation of Maltose by Shiga Dysentery Bacilli.

In the preceding description of the detection of glucose in the presence of maltose by acid fermentation, it was convenient to speak of the Shiga dysentery and atypical colon bacilli used for fermenting agents as "non-maltose-fermenting bacteria." Cultures of these strains made in maltose broth with the usual sizes of inoculum showed no reddening of Andrade indicator after 48 hours incubation. Although this would be sufficient to classify the strains as "non-maltose fermenters" in a routine study of intestinal bacilli, a gradual fermentation of maltose even under the routine conditions was indicated by a faint reddening of the indicator after 1 or 2 weeks storage of cultures at room temperature. In such cases, however, the question of maltose-fermenting capacity is complicated to some extent by the possibility of a gradual hydrolysis of maltose in dilute (1 per cent) solutions.

The question of the extent of maltose fermentation by Shiga dysentery bacilli assumed importance in determining the period of time that should be allowed for the incubation of the final fermentation mixtures of maltose and glucose with the fermenting agents. The mixture, obviously, should be incubated long enough to insure the complete fermentation of the glucose, but if maltose is slowly fermented the incubation period should not be extended too long or the acid formed from the larger amount of maltose will obscure the acid formation from the hexose. Even a slight capacity to produce acid from maltose, which, with the fewer number of cells in a routine broth culture, would result in only traces of acid, may result in a

very significant amount of acid in tests with the concentrated suspensions of bacilli used in our experiments.

Preliminary tests were made to determine the most desirable period of incubation to allow the maximum acid formation from glucose with the minimum acid production from the maltose. A typical experiment is outlined below.

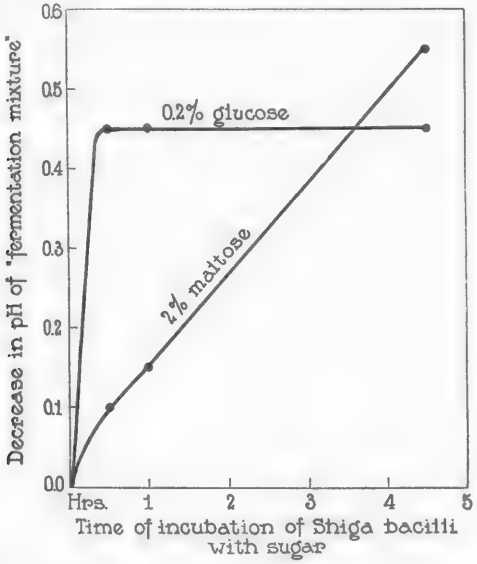

FIG. 1. "Slow fermentation" of maltose by Shiga dysentery bacilli. Relative rates of acid production by action of concentrated suspensions of the bacilli upon glucose and maltose.

Three tubes were prepared: (1) 5 cc. pH 7.6 phosphate solution plus 1.0 cc. sterile H_2O; (2) 5 cc. phosphate plus 1.0 cc. 20 per cent maltose solution; (3) 5 cc. phosphate plus 1.0 cc. 2 per cent glucose. To each of these tubes 3 cc. of concentrated suspension of Shiga dysentery bacilli were added. The final mixtures, therefore, represented "fermentation mixtures" of the same initial pH and the same buffer value containing respectively: (1) no sugar, (2) 2 per cent maltose, (3) 0.2 per cent glucose.

The mixtures were shaken and placed in the water bath. Samples were removed and pH measurements made after centrifugation at the end of 30 minutes, 1 hour, and 4 hours. The results are given in Fig. 1.

Fig. 1 illustrates the differences in rates of fermentation of glucose and maltose by our strain of Shiga bacilli. It is seen that glucose is fermented extremely rapidly, since the maximum acidity was reached within 30 minutes. Maltose, on the other hand, was fermented slowly as only a slight increase in acidity (0.1 pH) was evident at the time the maximum amount acid had been formed from the glucose. The continued, although gradual, increase in acidity in the 2 per cent maltose mixture (Fig. 1) emphasizes the necessity of avoiding prolonged incubation periods since it is obvious that the acid formed from traces of glucose would in time be obscured if an excess of maltose is present in the system.

From the relations revealed in this and other preliminary experiments it was concluded to adopt 45 minutes as the period of incubation of the test "fermentation mixtures." This period was greater than that required for the complete fermentation of the glucose with concentrated suspensions of the fermenting agents. In all cases, therefore, it was necessary to control the slight amount of acid produced from the maltose by the methods previously described.

All that is desired here is to establish the conditions for detection of glucose in the presence of maltose. Whether the "slow" fermentation of maltose is due to the presence of a small proportion of maltose-fermenting variants, or to a very slight maltose-fermenting capacity inherent in all dysentery bacilli, is of relatively little importance. A slight acid formation by Shiga bacilli in maltose solutions has been ascribed by some investigators to traces of glucose resulting from the heating of maltose during sterilization, or from a gradual hydrolysis in media which have been stored for a long time before use. Fig. 1, however, shows clearly that this explanation does not hold for the gradual fermentation of maltose by our strain. The differences in the form of the curves of acid production in the glucose and maltose can be due only to differences in rates of fermentation of the two sugars, since the curves would be similar in nature if the acid production in the "maltose solution" were due to traces of glucose. The above results, therefore, are also of importance as evidence that the production of small amounts of acid in maltose solutions by at least some strains of Shiga dysentery bacilli is a definite property which certainly cannot be explained by the presence of traces of glucose in the sterilized maltose.

Activity of Meningococcus Maltase and the Influence of Concentration of the Enzyme Solution upon Glucose Formation.

In the following experiment, the maltose-splitting activity of sterile, filtered solutions of meningococci is tested by the biological method just described. The relation of the degree of maltose hydrolysis to the concentration of the bacterial enzyme is demonstrated by tests made upon mixtures containing different concentrations of the meningococcus cell solution. The protocol of the experiment is summarized in Table I.

TABLE I.

Influence of Concentration of Enzyme upon Glucose Formation by Meningococcus Maltase.

Hydrolysis mixture — Amount of enzyme solution added to maltose solution		Hydrolysis mixture treated with the Fleming strain of colon bacilli	Hydrolysis mixture treated with Shiga dysentery bacilli	Change in pH due to fermentation of the glucose previously formed by meningococcus maltase enzyme (determined by fermentation with)	
		Unheated bacilli	Unheated bacilli	Fleming strain of colon bacilli	Shiga dysentery bacilli
cc.		*pH*	*pH*	*pH*	*pH*
2.0	Active enzyme	6.3	6.2	0.9	1.0
	Heat-inactivated enzyme	7.2	7.2		
1.0	Active enzyme	6.4	6.3	0.7	0.8
	Heat-inactivated enzyme	7.1	7.1		
0.5	Active enzyme	6.8	6.8	0.3	0.3
	Heat-inactivated enzyme	7.1	7.1		
0.2	Active enzyme	7.0	7.0	0.1	0.1
	Heat-inactivated enzyme	7.1	7.1		
0.1	Active enzyme	7.1	7.1	0.0	0.0
	Heat-inactivated enzyme	7.1	7.1		
0.0	(Control)	7.1	7.1	0.0	0.0

The results presented in Table I demonstrate that meningococci possess an enzyme which hydrolyzes maltose. The glucose formed

by the splitting of the maltose was detected by the formation of acid by glucose-fermenting bacteria. Too many factors are involved for one to attempt to show a precise quantitative relation between the different pH increments and the actual amount of glucose formed. However, the fact that the largest amounts of acid were formed in the mixtures in which the enzyme concentration was greatest, indicates that there existed a direct relation between the concentration of the meningococcus maltase and glucose formation.

TABLE II.

Comparison of Action of Meningococcus Enzyme Solution upon Sucrose, Raffinose, and Maltose as Indicated by Changes in Reactions.

Hydrolysis mixture		Change in pH due to fermentation of the hexose previously formed by meningococcus enzyme
		pH
Maltose	Active meningococcus enzyme solution	0.9
	Heat-inactivated meningococcus enzyme solution	0
Sucrose	Active meningococcus enzyme solution	0
	Heat-inactivated meningococcus enzyme solution	0
Raffinose	Active meningococcus enzyme solution	0
	Heat-inactivated meningococcus enzyme solution	0

Action of Meningococcus Enzyme Solution upon Sucrose and Raffinose.

The preceding experiment has demonstrated that meningococci possess a maltase enzyme. Since the method of demonstration of the maltase was indirect, it seemed important to apply the same biological method to solutions of sucrose and raffinose, neither of which is fermented by meningococci.

Solutions of sucrose and raffinose were prepared like the maltose solution. 2 cc. of active enzyme were added to 3 cc. of each of the sugar solutions; a second series was prepared with the same amount of heat-inactivated enzyme. The hydrolysis mixtures were incubated at 37°C. for 72 hours. The protocol of the experiment is summarized in Table II.

From Table II, it is seen that no acid is formed by hexose-fermenting Shiga bacilli when added to previously incubated mixtures of meningococcus enzymes and sucrose or raffinose solutions. This fact proves that the meningococcus cell solution hydrolyzes neither sucrose nor raffinose. Since it is commonly assumed that the inability of bacteria to ferment certain disaccharides or trisaccharides is due to their inability to hydrolyze the sugar, the above demon-

TABLE III.

Heat Lability of Meningococcus Maltase.

Hydrolysis mixture — Maltose solution plus meningococcus enzyme solution		Hydrolysis mixture treated with Shiga dysentery bacilli	Change in pH due to the fermentation of the glucose previously formed by action of the meningococcus maltase	
Treatment of enzyme solution	Amount of enzyme solution		2.0 cc. enzyme	1.0 cc. enzyme
	cc.	*pH*	*pH*	*pH*
Unheated	2.0	6.2	0.9	0.6
	1.0	6.4		
Heated 10 min. at 45°C.	2.0	6.6	0.5	0.3
	1.0	6.7		
Heated 10 min. at 50°C.	2.0	6.8	0.3	0.1
	1.0	6.9		
Heated 10 min. at 55°C.	2.0	7.1	0.0	0.0
	1.0	7.0		
Heated 10 min. at 60°C., 65°C.; 70°C.; 80°C.; 100°C.	2.0	7.1	0.0	0.0
	1.0	7.0		

strated absence of sucrase and raffinase is to be expected. However, the fact that significant increases in acidity occur only in the maltose solution treated with enzyme is important as evidence of the validity of the described biological method as a means of demonstrating a bacterial maltase enzyme.

Heat Lability of Meningococcus Maltase.

The following experiment was made to determine the relative degree of heat lability of meningococcus maltase.

Tubes of enzyme solution, sealed with vaseline to prevent oxidation at the higher temperatures, were immersed in a water bath that was constantly stirred, and heated for 10 minutes at the following temperatures: 45°, 50°, 55°, 60°, 65°, 70°, 80°, and 100°C. Samples of 2.0 cc. and 1.0 cc. of each of the heated enzyme solutions were added to separate tubes containing 3 cc. of 3 per cent maltose. The hydrolysis mixtures were incubated for 72 hours at 37°C. and were then tested for glucose by the fermentation method used in the preceding experiments. The protocol is summarized in Table III.

It is evident from the results of this experiment (Table III) that meningococcus maltase is entirely inactivated by 10 minutes exposure to 55°C. Since Table I has shown that the concentration of active enzyme is related to the amount of acid produced by the glucose-fermenting bacteria, differences in the final acidity offer a rough index of the relative amount of enzyme remaining active after the various heatings. When analyzed from this standpoint, the above results show that the enzyme is destroyed to some extent when heated to temperatures lower than 55°C. A significant loss in activity occurs during 10 minutes exposure at 45°C., and only a small proportion of the maltase remains active after 10 minutes at 50°C. Thus, this enzyme, like the carbohydrate-hydrolyzing enzymes of pneumococci (4) is an extremely heat-labile substance.

Endocellular Nature of Meningococcus Maltase.

The method of preparation of the meningococcus derivatives which we have studied indicates that the maltase is an endocellular substance freed when the bacterial cell is disrupted. However, to obtain actual proof of its endocellular nature the following comparison was made of the maltase activity of the supernatant fluid of a broth culture with the activity of a solution prepared from the centrifuged cells of the same culture.

1 liter of a 24 hour yeast extract broth culture of meningococci was centrifuged, and the supernatant removed from the bacterial sediment.

The meningococcus cells were collected and subjected to the freezing and thawing process. Both the supernatant fluid and the cell solution were finally filtered through a Berkefeld filter. The comparison of the maltase activity of the two preparations was made by the procedure used in the preceding experiments.

The results are summarized in Table IV.

The results of this experiment (Table IV) prove that meningococcus maltase is an endocellular substance, since maltase activity is exhibited by sterile solutions prepared from the bacterial cells and not by the supernatant or filtrate of the unautolyzed broth

TABLE IV.

Endocellular Nature of Meningococcus Maltase.

Hydrolysis test mixture	Change in pH due to fermentation of glucose formed by meningococcus maltase	Maltase activity
Maltose plus filtered solution of meningococcus cells	0.8	+
Maltose plus filtrate of broth culture of meningococcus	0.0	−

culture. Needless to say, the endocellular nature of the enzyme does not preclude the possibility of a demonstration of maltase activity by filtrates of broth cultures in which considerable cell autolysis has occurred (5).

DISCUSSION.

Studies on the enzymes of pathogenic bacteria are of more than academic interest. The life and growth of the bacteria, either in artificial culture or in the infected host, are dependent upon their metabolic functions, most of which in turn depend upon the action of enzymes. Knowledge concerning the enzymes leads to a better understanding of the mechanism of the biochemical processes exhibited in the test-tube and of the changes accompanying the growth of the bacteria in the body and the factors concerned in the satisfaction of their nutritional needs. The endocellular nature of many of the enzymes introduces a further point of interest since these enzymes must be considered as actual constituents of the bacterial cell which, in the case of pathogenic microorganisms, would be liberated into the tissues of the host if lysis of the bacteria occurs at any stage of the infection.

The results of the experiments presented in this paper demonstrate the maltose-hydrolyzing activity of filtered solutions of the intracel-

lular substances of meningococci. Hence, the splitting of maltose, the first step in the acid fermentation of this sugar by living meningococci, can be referred to a maltase enzyme the activity of which is independent of the presence of intact bacterial cells. The amount of maltose hydrolysis effected by the meningococcus maltase bears a direct relationship to the concentration of the bacterial enzyme. The maltase of meningococci, like the carbohydrate-splitting enzymes of pneumococci, is an extremely heat-labile substance. It is inactivated by prolonged exposure to 45°C., and is entirely destroyed by a short exposure to 55°C.

SUMMARY.

Meningococci possess an endocellular maltase enzyme. The splitting of maltose by this enzyme has been demonstrated by a biological method based upon the acid fermentation of glucose by bacteria which have little or no action upon maltose.

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STUDIES ON BACTERIAL ENZYMES.

II. Meningococcus Peptonase.

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INTRODUCTION.

The present paper reports a study of the peptone-hydrolyzing enzyme of Meningococcus. The activity of this enzyme has been studied with sterile solutions of the bacterial substances, not only devoid of living bacteria but freed of bacterial cells by Berkefeld filtration.

The maltase of Meningococcus has been demonstrated in the preceding paper (1). The fragility of meningococci and the readiness with which they undergo dissolution, facilitate the preparation of sterile solutions of their intracellular substances. A search of the literature, however, has revealed little work on the enzymes of Meningococcus. Flexner (2) in his early studies on Meningococcus demonstrated experimentally the enzymatic nature of the processes involved in the dissolution of the bacteria which occurs in laboratory cultures. He recognized the intracellular nature of the autolytic enzyme and studied the conditions which influence its operation. Since that time, there have been numbers of incidental reports on the autolysis of meningococci without, however, evidence referring the process to a bacteriolytic enzyme. Sturges and Rettger (3) studied the autolysis of suspensions of meningococci and observed that increases in amino nitrogen accompanied the morphological disintegration of the bacterial cells. Their paper is concerned, however, with autolysis of bacterial suspensions and does not furnish a demonstration of an active enzyme operative in solutions filtered free from bacteria.

EXPERIMENTAL.

Methods.

Preparation of Enzyme Solutions.—The earliest reports on bacterial enzymes were concerned with the so called "exocellular" enzymes which are liberated or

secreted into the culture medium during the growth of the bacteria. It is now recognized, however, that many of the enzymes of bacteria are more intimately bound to the microbial cell and are set free into the surrounding medium only upon its disintegration and the experimental demonstration of these enzymes depends upon their liberation either by imposed physical or chemical conditions or by autolytic processes. Since the enzymes are labile substances, it is important to prevent their deterioration or inactivation during the processes mentioned. Studies (3) on the oxidation of pneumococcus enzymes present many examples of the importance of protection of enzyme solutions from air during their preparation.

The meningococcus enzyme solutions used in this study were derived from a strain of normal meningococci and were prepared by the method described in the preceding paper (1).

Substrates.—2 per cent solutions of Bacto-peptone or proteose peptone were prepared in 0.1 M phosphate solution (pH 7.6). The peptone solutions were sterilized in the autoclave.

TABLE I.

Hydrolysis of Peptone by Sterile Solutions of Meningococcus.

NH_2-N per 100 cc. of hydrolysis mixture		
Heat-inactivated enzyme	Active enzyme	Increase due to action of enzyme
mg.	*mg.*	*mg.*
34.0	46.5	12.5

Amino Nitrogen Determinations.—The increases in amino nitrogen were determined by direct analysis of the enzyme-substrate mixtures by Van Slyke's nitrous acid method.

Sterility Control.—No antiseptics were added to any of the hydrolysis mixtures. Sterility was controlled by cultural methods.

Action of Enzymes of Meningococcus upon Peptone.

The first step in the investigation consisted in tests of the action of the sterile solutions of meningococci upon peptone. It is unnecessary to describe these experiments since the protocol given in Table I is sufficient evidence of the presence of an active enzyme which hydrolyzes peptone in the complete absence of living or formed bacterial cells.

In the experiment reported in Table I, 0.5 cc. of the active enzyme solution was added to 5.0 cc. of sterile peptone solution; an equal amount of enzyme previously inactivated by heat was added to another tube of the peptone substrate to serve as control of the active enzyme. After 36 hours incubation, amino nitrogen analyses were made to determine the extent of peptone hydrolysis. The protocol is given in Table I.

The protocol given in Table I is evidence of the active enzyme of Meningococcus which retains its ability to hydrolyze peptone in the complete absence of living or formed bacterial cells.

Heat Lability of the Peptonase Enzyme of Meningococcus.

All of the usual bacterial enzymes are sensitive to heat. The following experiment investigated the degree of thermolability of the peptonase enzyme of Meningococcus.

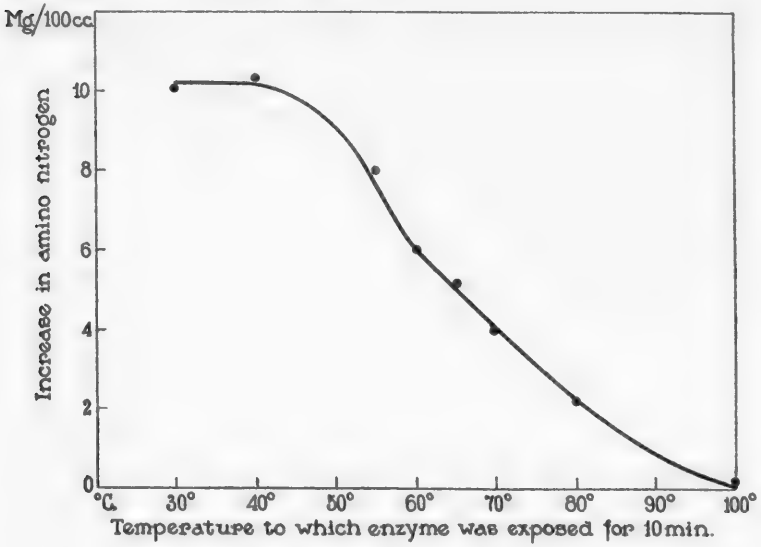

FIG. 1. Heat lability of meningococcus peptonase.

Portions of the enzyme solution (pH 7.4) were heated for 10 minutes at 40°, 55°, 65°, 80°, and 100°C. Equal amounts of the heated enzyme were added to a series of tubes containing 5 cc. of sterile peptone solution. A control tube of peptone to which unheated enzyme was added was used to determine the initial activity of the enzyme. The results of the experiment are presented in Fig. 1.

As shown in Fig. 1, the endocellular peptonase enzyme of Meningococcus is a heat-labile substance. The rate of its inactivation in-

creases with increase in temperature, and it is entirely destroyed by exposure to 100°C. for 10 minutes.

Intracellular Nature of Peptone-Splitting Enzymes of Meningococci.

The fact that the active enzymes demonstrated by the experiment yielding the figures given in Table I were prepared by disruption of the bacteria, in itself indicates that the peptone-hydrolyzing enzyme is of endocellular nature. Proof of this was obtained by comparing the peptone-splitting activity of the supernatant of an unautolyzed broth culture with the activity of a sterile solution prepared from the bacterial cells removed from the broth culture by centrifugation. The results of these experiments showed that while solutions of the intracellular substances of the bacteria actively hydrolyzed peptone, the bacteria-free supernatant fluid of unautolyzed cultures is practically devoid of peptone-splitting properties.

A slight activity exhibited by the bacteria-free filtrate of the broth culture can be referred to the liberation of the intracellular substances by the dissolution of the bacterial cells, a process which occurs relatively early in meningococcus cultures.

Comparison of Resistance to Heat and to Oxidation of the Maltase and Peptonase Enzymes of Meningococci.

In a previous paper on the oxidation of pneumococcus enzymes (4) it was pointed out that the activity of some of the more labile enzymes may be destroyed by oxidation processes which cause little loss in the activity of the more resistant enzymes present in the same mixture of intracellular substances. It was also shown that the relative order of resistance of the hydrolyzing enzymes to oxidation agrees with the order of heat resistance of the same enzymes. It seemed of interest, therefore, to determine if the same relation holds true for the maltase and peptonase enzymes of meningococci, both of which are present in the bacterial cell solution.

Facts illustrating the relative resistance of the meningococcus enzymes were obtained by the following experiment.

A portion of enzyme solution was heated for 10 minutes at 55°C., a period which had previously been proved sufficient to completely inactivate the maltase. Two

other portions of enzyme solution were exposed for 12 hours to different concentrations of H_2O_2. The initial concentration of H_2O_2 in the first tube was 150 mM; in the second tube, 25 mM. A part of the H_2O_2 was destroyed by the catalase and peroxidase of the meningococcus cell solution so that the concentration of the oxidizing agent during most of the oxidation treatment was considerably less than is represented by these figures.

For tests of peptone-splitting activity the following hydrolysis mixtures were prepared: (1) peptone plus untreated, or active enzyme; (2) peptone plus enzyme heated 10 minutes at 55°C.; (3) peptone plus enzyme exposed to 25 mM H_2O_2; (4) peptone plus enzyme exposed to 150 mM H_2O_2; (5) peptone plus enzyme completely inactivated by heat (boiled). A similar series was prepared to test the maltase activity, which was done by adding equal amounts of the above treated enzyme solutions to sterile maltose.

TABLE II.

Relative Resistance to Heat and to Oxidation Exhibited by the Peptonase and Maltase Enzymes of Meningococcus.

Treatment of enzyme	Peptonase activity (increase in NH_2-N, mg. in 100 cc.)	Maltase activity
	mg.	
Active (or untreated) enzyme solution	13.0	+
Enzyme solution heated 10 min. at 55°C.	9.8	0
Enzyme solution exposed to 25 mM H_2O_2	10.2	0
Enzyme solution exposed to 150 mM H_2O_2	3.1	0
Enzyme solution heated 30 min. at 100°C. (control)	0	0

The maltose-splitting was determined by the biological method described in the preceding paper.

The results of this experiment (Table II) show that the meningococcus maltase is completely inactivated by heating and oxidation treatment which is insufficient to inactivate all of the peptone-hydrolyzing enzyme. This difference in relative stability of the carbohydrate-splitting and peptone-splitting enzymes of meningococci is similar to that previously shown (3) to hold for the same enzymes of pneumococci.

DISCUSSION.

Evidence has been presented that the meningococcus cell contains an active enzyme which on separation from the bacteria suffices to

hydrolyze peptone to simpler peptides and amino nitrogen. The commercial peptone used as substrate in the demonstration of the enzyme represents a mixture of protein hydrolysis products which includes derivatives of varying degrees of complexity. Since living cultures of meningococci do not exhibit the marked proteolytic properties possessed by the so called "liquefying" bacteria, it is probable that the enzyme solutions used in the present investigation would not attack intact or native proteins to the same degree as peptone. In view of this evidence that the sterile bacterial solution acts more readily on the simple protein derivatives occurring in "peptone," it is advisable to adopt the term "peptonase" in referring to the active enzyme involved. This term has been employed by Avery and Cullen (5) to describe the enzyme or enzymes of Pneumococcus which hydrolyze the peptides of peptone to simpler products. The "peptonase" enzyme of Meningococcus is more resistant both to heat and to oxidation than is the maltase enzyme, just as the "peptonase" enzyme of Pneumococcus is more resistant than the carbohydrate-splitting enzymes of the same bacteria.

The peptonase enzyme is endocellular in nature and must be regarded as a heat-labile constituent of the meningococcus cell, which would be set free whenever lysis of the bacteria occurs either in artificial culture or in the tissues of an infected host.

SUMMARY.

The meningococcus cell contains a peptonase enzyme which hydrolyzes the peptides and similar constituents of commercial "peptone." The activity of this enzyme is independent of the presence of the formed bacterial cell. The peptonase enzyme is more resistant to heat and to oxidation than is the maltase enzyme of the same bacteria.

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STUDIES ON BACTERIAL ENZYMES.

III. Pneumococcus Maltase and Lactase.

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INTRODUCTION.

Intensive study of Pneumococcus at the Hospital of The Rockefeller Institute has resulted in a better understanding of the constituents of the bacterial cell. Among the active substances thus far studied may be mentioned, the endocellular hemotoxin (1), proteolytic enzymes (2), lipolytic enzymes (2), carbohydrate-splitting enzymes (2), bacteriolytic enzymes (2), and thermolabile cellular substances (3) concerned in oxidation and reduction processes; together with the carbohydrate soluble specific substance, and the antigenic protein fraction of the cell (4).

The carbohydrate-hydrolyzing enzymes previously recognized include the amylase, inulinase, and sucrase reported by Avery and Cullen (2) and the raffinase reported by Neill and Avery (5). The experiments reported here add maltase and lactase enzymes to the list of agents involved in the biochemical activities of living pneumococci.

While, as a general principle, it is true that hydrolysis to hexoses is a preliminary step in the acid fermentation of lactose and similar disaccharides by the more common bacteria, the assumption that this applies to all bacteria is based upon scant experimental evidence, and some authorities (5) (Kruse, Fischer, and others) have concluded that the rule is not universally applicable. A number of workers (6) have shown that the products of the fermentation of complex carbohydrates differ sometimes not only quantitatively, but qualitatively from those yielded in the fermentation of the component hexoses. Gayon and Dubourg (7) report differences in the products of the fermentation of

sucrose and of fructose which are especially difficult to explain if sucrose is hydrolyzed before being fermented by the types of bacteria they employed. Phenomena of this nature have been observed chiefly in "mixed acid" fermentations rather than in the type of acid fermentation induced by the "true" lactic acid bacteria most closely related to Pneumococcus. However, the existence of microorganisms inducing an unusual type of fermentation of the complex sugars makes it worth while to seek for experimental evidence of the hydrolyzing enzymes rather than to assume their presence from the fact that the disaccharide is fermented by the bacteria.

TABLE I.

Demonstration of Activity of Pneumococcus Maltase and Lactase.

Hydrolysis mixture		Hydrolysis mixture treated with		Change in pH due to fermentation of hexoses formed by pneumococcus enzymes
		Shiga dysentery bacilli	Typhoid bacilli	
		*p*H	*p*H	*p*H
Maltose	Active enzyme	7.0	No test	0.5
	Heat-inactivated enzyme	7.5	No test	
Lactose	Active enzyme	6.5	6.6	1.0
	Heat-inactivated enzyme	7.5	7.5	

EXPERIMENTAL.

Methods.

Enzyme Solutions.—Sterile extracts of pneumococcus cells were used to demonstrate the enzymes. The pneumococcus extracts were prepared by the method previously described (3). Broth cultures were centrifuged; the concentrated bacterial cells were collected and suspended in a small volume of the supernatant broth; the pneumococcus suspensions were frozen and thawed repeatedly to liberate the intracellular substances. The extracts were finally filtered through a Berkefeld candle. Hence, the pneumococcus extracts employed as enzyme solutions were not only sterile but were free from cell fragments.

Demonstration of Activity of Pneumococcus Maltase and Lactase.

The following experiment illustrates the fact that sterile extracts of pneumococci possess the property of hydrolyzing maltose and lactose.

0.2 cc. of a sterile solution of pneumococci was added to 3.0 cc. of sterile maltose and lactose solution. A control series was prepared with enzyme solution which had been inactivated by heat. Shiga dysentery bacilli, which attack neither maltose nor lactose, were used as fermenting agents in the detection of the hexose products of the enzyme action. Tests of the lactose hydrolysis mixtures were also made with typhoid bacilli.

The results given in Table I demonstrate that pneumococci possess an active maltase and an active lactase enzyme in addition to the previously described carbohydrate-splitting enzymes. It is interesting to note that the lactase enzyme is apparently more active than the maltase as indicated by the greater acid formation in the fermentation tests made upon the lactose hydrolysis mixture. Since the concentration of the pneumococcus solution was the same in both the maltose and lactose tests, this fact suggests that either the pneumococcus cell contains a more active lactase, or that the maltase enzyme if initially as active had deteriorated to a greater extent than the lactase at the time of the enzyme tests.

Comparison of the Activity of Different Carbohydrate-Splitting Enzymes of Pneumococcus.

The preceding experiment showed an apparent difference in the activity with which the sterile pneumococcus cell solution hydrolyzed maltose and lactose. It, therefore, seemed of interest to compare the apparent activity of other carbohydrate-splitting enzymes contained in the sterile cell solution with the activity of the maltase.

This comparison was made by the fermentation method used in the preceding experiment. Equal amounts of the pneumococcus solution were added to sterile solutions of maltose, lactose, sucrose, and raffinose. The hexose products of the enzyme action were detected by use of Shiga dysentery bacilli as fermenting agents. There is an objection to this method in such comparisons since the tests may involve differences in the ease of fermentation of the different hexoses (glucose, fructose, and galactose) yielded in the hydrolysis of the different sugars. However, these differences are almost always simply differences in rate of acid formation, which vanish when a greater time is allowed for hexose fermentation by the dysentery bacilli. The results of the experiment are summarized in Table II.

Without presuming to attach too precise a quantitative value to the data in Table II, one can yet be certain that the differences in acid

formation in the test solutions indicate differences in the degree to which the various sugars have been hydrolyzed by the pneumococcus enzymes. The acid produced in the maltose mixture was unquestionably much less in quantity than that in any of the other test solutions. This indicates that the pneumococcus cell solution hydrolyzes maltose less actively than either of the other two disaccharides, and, more surprising, less actively than the trisaccharide, raffinose. One cannot be sure whether these experimental results are due to a relative inability of pneumococcus cells of this particular strain to hydrolyze maltose or

TABLE II.

Comparison of the Activity of Different Carbohydrate-Splitting Enzymes of Pneumococcus.

Hydrolysis mixture		Change in pH due to acid fermentation of hexose formed by pneumococcus enzymes
Test sugar	Amount of pneumococcus cell solution	
		pH
Maltose	0.4 cc. enzyme solution	0.5
	0.1 cc. enzyme solution	0.3
Lactose	0.4 cc. enzyme solution	1.7
	0.1 cc. enzyme solution	0.6
Sucrose	0.4 cc. enzyme solution	1.4
	0.1 cc. enzyme solution	0.6
Raffinose	0.4 cc. enzyme solution	1.0
	0.1 cc. enzyme solution	0.5

whether the maltase enzyme is especially labile and is destroyed more readily during the manipulation involved in the preparation of the enzyme solution. Maltose is apparently very readily hydrolyzed by the intact cells, for it seems to be as rapidly fermented as lactose in tests with the living bacteria.

Comparative tests of the heat lability and of the resistance to oxidation of the maltase, lactase, and sucrase were made on the enzyme solutions. In all such tests, the cell solution lost its maltase activity entirely after a heating and oxidation treatment which was insufficient to destroy all of the active lactase and sucrase. The

results, however, were not conclusive since they were complicated by the greater initial activity of the lactase and sucrase in the original cellular solution.

DISCUSSION.

Experimental proof of most of the microbial carbohydrases has been made with yeasts and fungi, although sucrase has been described from a number of different bacteria. An extended search of the literature, however, reveals very few actual demonstrations of active maltase and lactase enzymes in sterile solutions prepared from bacteria. The results of the experiments described prove the existence of active lactase and maltase in sterile, filtered solutions of the intracellular substances of pneumococci. The recognition of these two enzymes adds to the list of cellular substances known to be involved in the metabolic functions of living pneumococci.

The pneumococcus is closely related to the saprophytic streptococci involved in the common souring of milk. Microbiologists (Jensen (9), and others) who are interested primarily in the biochemical activities of bacteria point out the fact that pneumococci and the lactic streptococci (*Streptococcus lacticus*) possess sufficient characters in common to justify their inclusion in the same biological group of "lactic acid streptococci." The lactic acid fermentation of lactose and other disaccharides is generally believed to involve two reactions: (1) the hydrolysis of the disaccharide to its component hexoses; (2) the conversion of the hexoses to lactic acid. With living cells it is impossible to demonstrate the products of the first reaction as only the final acid product can be detected. Like the intermediate products of many chemical reactions, the hexoses never accumulate in detectable amount, since the living bacteria convert them to acid as rapidly as they are formed from the disaccharide. Indeed, the actual proof that two reactions are involved rests upon the fact that of the two processes (hydrolysis and fermentation) effected by the living cell, only the hydrolytic property retains its activity in sterile filtered solutions of the intracellular substances. Apparently, the power of the bacterial cell to attack hexoses is most intimately bound up with its morphological integrity, since solutions of the cellular substances, although

exhibiting many of the activities of the intact bacteria, seem to be devoid of action upon glucose (9).

Pneumococcus, in its present state of adaptation, is generally considered to have no extended independent existence outside of the animal body and its association with carbohydrates would seem to be limited to the foodstuffs encountered during its growth in the mouth. It is interesting, therefore, to find that the strictly parasitic Pneumococcus contains an endocellular equipment of carbohydrases that rivals the generous endowment of purely saprophytic fungi.

SUMMARY.

Lactase and maltase are constituents of the pneumococcus cell.

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THE ETIOLOGY OF VERRUGA PERUANA.

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PLATES 2 TO 4.

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The experimental investigation to be reported in this paper was made possible through the cooperation of Professor Oswaldo Hercelles, of the University of Lima, who was kind enough to obtain for me selected material for study, and I wish at the outset to acknowledge my indebtedness to him and my appreciation of his courtesy. I wish to express my thanks also to Professor E. Campodónico, of the University of Lima, through whose courtesy the material was forwarded from Lima to New York.

There are many similarities between the conditions known as verruga peruana and Oroya fever. Their geographical distribution, which is rather curiously limited to certain narrow valleys on the western slopes of the Andes Mountains, between 6° and 13° south latitude, is practically the same and is of the type shown by infections conveyed by certain ectoparasites. Fever and anemia are considerably more acute and severe in Oroya fever, but both are present in verruga. Verruga is predominantly a chronic infection, and its conspicuous feature is the characteristic nodular eruption on the skin which lasts for a period of a few weeks to one of many months. Oroya fever, on the other hand, while sometimes accompanied or followed by a similar skin eruption, is distinguished by an acute course of extreme anemia, during which a specific intracellular parasite, *Bartonella bacilliformis*, is abundantly present in the red blood corpuscles. This microorganism has rarely been seen in the blood in cases of benign verruga.

In 1910 Jadassohn and Seiffert[1] showed that verruga peruana could be transmitted to monkeys by local inoculation of suspensions

[1] Jadassohn and Seiffert, G., *Z. Hyg. u. Infectionskrankh.*, 1910, lxvi, 247.

of tissue from human verruga lesions, and their results have received confirmation from Mayer, Rocha-Lima, and Werner,[2] and from the Commission of the Harvard School of Tropical Medicine.[3]

Oroya fever has not thus far been directly transmitted to animals, but by means of a culture recently isolated from a fatal case of the disease[4] it has been shown that young *rhesus* monkeys (about 2,000 gm. body weight) are susceptible to infection with *Bartonella bacilliformis*, though usually not to the same degree as man, and that in the infected animals the parasite is demonstrable in its characteristic situation in the erythrocytes. In some animals which are less resistant to infection, the extreme anemia of Oroya fever has been reproduced.[5] The striking fact brought out in the inoculation experiments, however, was the dual nature of the infection induced, for characteristic signs of both Oroya fever and verruga were observed in the animals, with occasionally a marked favoring of one or the other type of disease, as the virulence of the strain became enhanced by adaptation to the experimental animal. Systemic manifestations—moderate or marked anemia, fever, and high titer of *Bartonella bacilliformis* in the blood, bone marrow, lymph nodes, and spleen—were in general more severe in animals inoculated intravenously or intraperitoneally, and death occurred only in animals so inoculated, but the invasion of the blood by the parasite was found to take place also after local inoculation. The tendency toward the production of verrucous lesions was more marked, as a rule, when the virus was introduced locally, and the lesions usually arose only at the sites of inoculation, as is the case when monkeys are inoculated directly with human verruga material.[1,2] In one instance, however, in which passage virus was injected both locally and intravenously, a spontaneous general eruption typical of severe human verruga arose on various parts of the skin, remote from the sites of inoculation. The skin lesions of the animals, whether local or

[2] Mayer, M., Rocha-Lima, H., and Werner, H., *Münch. med. Woch.*, 1913, lx, 739.

[3] Strong, R. P., Tyzzer, E. E., Sellards, A. W., Brues, C. T., and Gastiaburú, J. C., Report of first expedition to South America, 1913, Harvard School of Tropical Medicine, Cambridge, 1915.

[4] Noguchi, H., and Battistini, T. S., *J. Exp. Med.*, 1926, xliii, 851.

[5] Noguchi, H., *J. Exp. Med.*, 1926, xliv, 697.

spontaneous in occurrence, in all instances yielded cultures of *Bartonella bacilliformis*, and stained sections of all such lesions revealed the parasites in large numbers in a characteristic endothelial situation.[5]

These findings, when taken in relation to the historical and epidemiological data with regard to the two conditions,[6] suggested very strongly that Oroya fever and verruga peruana were both due to *Bartonella bacilliformis*, the considerable variations in the manifestations of infection being a result of the differences in resistance among individuals or among different tissues of the same individual. It was desirable, however, to approach the problem of the etiology of verruga directly, by means of the cultural and experimental methods used for the isolation of *Bartonella bacilliformis* from Oroya fever.

For the purpose of comparative study subcutaneous nodules were removed from each of two cases of verruga in the Dos de Mayo Hospital, Lima, placed in tubes, and covered with citrate-saline solution (2 per cent sodium citrate in 0.9 per cent sodium chloride). The tubes were sealed, abundant air space being allowed, and were placed in the ship's refrigerator during the journey to New York, a period of 14 days. We were fortunate in having transportation so arranged that the tissues were shipped shortly after their excision from the patients and were received in the laboratory the same day that they arrived in New York (April 7, 1926). Each specimen was immediately examined microscopically, saline suspensions were made, and inoculated into culture media and experimental animals, and a portion of each tissue was fixed in Regaud's fluid for histological study.

The nodule of Case P. 5 measured about 8 × 12 × 6 mm. and was light pinkish in color, with the exception of a small portion to which the skin was still attached. The nodule was still firm, except for slight softening along the cut surfaces. The citrate solution was slightly turbid, owing to liberation of tissue elements, but there was no putrefactive odor.

Minute Gram-negative bacilli were present, occurring singly, in pairs, and in larger groups, as well as in masses (Fig. 18). Sections stained with Giemsa's and Gram's solutions showed masses of the organisms scattered through the tissue (Fig. 18), the general structure of which was still well preserved (Fig. 17) notwithstanding loss of cellular elements through autolysis.

[6] Odriozola, E., La maladie de Carrion, Paris, 1896.

The nodule from Case J. 45 was not as well preserved. The tissue was soft and friable, but there was no sign of bacterial putrefaction. It contained numerous Gram-negative bacilli similar to those found in the specimen from Case P. 5 and in addition a few small Gram-positive diplococci. In sections the bacilli were found in the nodular tissue and the cocci near and on the skin.

Two species of minute Gram-negative microorganisms were isolated from the tissue of Case P. 5, one indirectly, from monkeys inoculated with suspensions of the tissue, and one by direct cultivation. The former is pathogenic for young *Macacus rhesus* monkeys and in every respect has proved identical with the strain of *Bartonella bacilliformis* previously obtained from the blood of a case of Oroya fever. The latter, although present also in the J. 45 tissue, as shown by cultivation experiments, is apparently a non-specific invader; it has failed to induce characteristic local or systemic infection, and it grows within 24 hours on ordinary agar slants, while the pathogenic organism grows slowly and only on leptospira medium or blood agar slants. Our interest, therefore, is in the pathogenic strain obtained by inoculation of the P. 5 material.

Transmission of Verruga Peruana to Monkeys with the Excised Nodular Tissue of Case P. 5.

Transmission of verruga peruana to monkeys, as stated earlier in this paper, has repeatedly been accomplished by previous investigators. In the present instance, however, the tissue had been excised from the patients more than 2 weeks previous to inoculation.

One young and one adult *Macacus rhesus* were available on April 7. The young one was used for inoculation of the more promising material, that is, the P. 5 nodule. Another young monkey, obtained on April 10, was also inoculated with the P. 5 material. Owing to the scarcity of suitable animals, the inoculations with the J. 45 material could not be repeated.

The suspension of the P. 5 nodule induced definite systemic or local infection in both animals inoculated; the full grown monkey receiving inoculation of the J. 45 material showed no symptoms of infection.

M. rhesus 33, inoculated Apr. 7, 1926, with a saline suspension of the nodule P. 5, intradermally on the shaved right eyebrow and by scarification on the left eyebrow.

2 cc. of the suspension were also injected into the saphenous vein of the right leg. After 48 hours there was a rise of temperature (Chart 1) which lasted for 2 days, but blood taken at this time yielded no growth. On Apr. 14 the temperature rose again and remained high for 6 days. Blood taken during the fever (Apr 16) and inoculated into leptospira medium yielded, in a 1:10 dilution, pure growth of a microorganism indistinguishable from *Bartonella bacilliformis*. The sites of intradermal inoculations on the eyebrow showed some induration at this time, but they never increased in size and receded within a few weeks. The lymph glands in the inguinal and axillary regions became markedly enlarged about Apr. 16. Blood cultures made on two successive occasions, May 27 and June 20, 1926, both gave pure cultures of the *Bartonella*-like organisms.

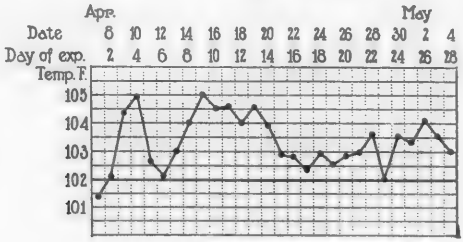


CHART 1. *M. rhesus* 33.

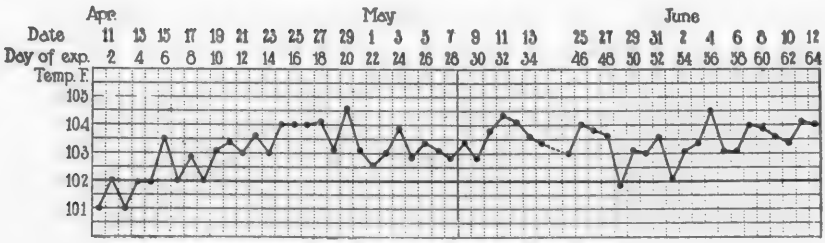


CHART 2. *M. rhesus* 34.

M. rhesus 34, inoculated Apr. 10, 1926, with the same material and in the same way as *M. rhesus* 33. A moderate rise of temperature occurred 14 days from the time of inoculation (Chart 2), and the animal remained febrile for 4 days. Blood cultures made on Apr. 28, 1926, yielded a pure growth of the same organism as had been isolated from *M. rhesus* 33. At this time the lymph glands had become markedly enlarged, but no local reactions were noticeable. Blood taken on May 12 and on May 27 was culturally positive in a 1:10 dilution. At the beginning of June a large subcutaneous nodule at the middle portion of the tail was noticed

(Fig. 10), and on June 18 it was excised.* It showed the typical histology of verruga tissue (Fig. 11) and yielded a pure culture of the same organism as had been obtained from the blood. Cultivation of the blood was again successful on June 21, this time with a dilution of 1:100.

The foregoing experiments demonstrate the infectivity of the verruga nodule which had been kept at refrigerator temperature for at least 2 weeks, and the association of infective power (as evidenced by febrile reaction and the production of a metastatic local verruga lesion) with a definite microorganism recoverable by culture both from the blood of the animals and from the local lesion. Blood counts revealed no anemia in either animal, and in neither instance did the blood films contain a sufficient number of organisms to be detected microscopically.

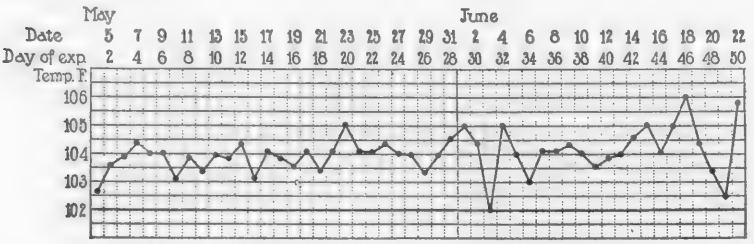


CHART 3. *M. rhesus* 41.

The pathogenicity of the culture obtained from the blood of *M. rhesus* 33 on April 16 was tested by inoculation of *M. rhesus* 41. As the protocol shows, the culture gave rise not only to a local reaction but to a systemic infection of pronounced severity.

M. rhesus 41 (Fig. 1) inoculated May 4, 1926, with culture derived from the blood of *M. rhesus* 33 and grown for 18 days on leptospira medium. The material was inoculated on the right eyebrow and abdomen intradermally and on the left eyebrow and abdomen by scarification. An irregular remittent febrile reaction (Chart 3) began to be manifest 72 hours from the time of inoculation and continued during the 2 months of observation. Induration became noticeable at the site of intradermal inoculation on the right eyebrow after 14 days. The lesion had the gross appearance of a typical verruga nodule, and histological examination of the

*All operations were carried out under ether anesthesia.

excised tissue showed the characteristic granulomatous structure (Figs. 4, 12) and the presence of bacilliform organisms in the endothelial cells (Fig. 13). A suspension of the nodular tissue yielded pure cultures of the organisms in a 1:10 dilution, as did also blood withdrawn on May 21. On May 27 the blood titer by culture was 1:10,000, and on June 3, 1:100,000. The nodules on the eyebrow rapidly increased in size, becoming scarlet-red and protruding (Fig. 1). Blood films made on June 3 showed numerous bacilliform bodies in the red corpuscles, the organisms being present in the largest numbers so far observed in *rhesus* monkeys (Figs. 2, 14). Small nodules developed at the sites of intradermal inoculation on the abdomen 40 days after inoculation; at that time the nodules on the eyebrow measured about 10 × 12 mm.

Blood counts made by Dr. J. H. Bauer showed a gradual diminution of red corpuscles and hemoglobin and an unusually high leucocytosis:

	Erythrocytes per c.mm.	Hemoglobin	Leucocytes per c.mm.
		per cent	
May 4 (day of inoculation).....	5,088,000	80	
" 14 (10 days after inoculation).....	4,632,000	70	
June 3 (30 " " ").....	4,760,000	55	17,600
" 15 (42 " " ").....	4,800,000	40	23,000
" 23 (50 " " ").....	3,392,000	30	43,000
" 25 (52 " " ").....	3,460,000	25	40,000
" 28 (55 " " ").....	3,552,000	25	37,800

The reduction in hemoglobin, it will be noted, was relatively greater than that in the number of red cells, a phenomenon so far not observed by us in animals infected with the strain of *Bartonella bacilliformis* from Oroya fever.

Blood smears stained on June 25 revealed a peculiar appearance of the intracorpuscular parasites, which were still present in rather large numbers, but stained reddish rather than violet, appeared thinner, and in some instances were fragmented. The general features of the organisms suggested that they were undergoing degeneration (Figs. 3, 15). Blood taken at this time yielded cultures in dilution of 1:10, but not in 1:100. A week later no intracorpuscular parasites could be demonstrated, the animal was more active, and the nodules had decreased somewhat in size. Conditions seemed to indicate the development of a state of immunity. No cultures could be obtained from the blood after June 25. On Aug. 11 there was still anemia (red blood cells 3,362,000, hemoglobin 50 per cent), but on Sept. 14 the number of red cells had increased to 4,336,000.

The nodule excised on May 19 and the blood withdrawn 2 days later were inoculated into two *rhesus* monkeys. As the protocols show, both materials gave rise to local lesions and also to systemic infection.

M. rhesus 42, inoculated May 19, 1926, with a saline suspension of the nodule from the eyebrow of *M. rhesus* 41, on the left eyebrow and left abdominal wall by intradermal injection and on the right eyebrow and right abdominal wall by scarification. This animal had fever for only a few days (Chart 4) during the 45 days of observation, but cultures made with the blood on June 2 and 14, 1926, gave pure growth of the bacilliform organisms in dilutions of 1:10 and 1:1,000 respectively.

Small reddish indurated areas along the lines of scarification on both eyebrow and abdomen became noticeable on June 1 (Fig. 6), and 10 days later the scarified areas appeared as linear rows of eruptions (Figs. 7, 8). Within another 10 days the adjacent lesions had become confluent (Fig. 9) but the transverse lines were still well separated. The intradermal inoculations in this instance, contrary to the usual case, failed to induce any lesions.

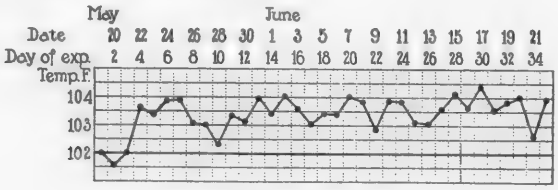


CHART 4. *M. rhesus* 42.

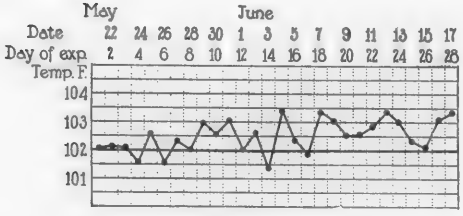


CHART 5. *M. rhesus* 43.

The lesions had all healed by Aug. 6, 1926.

M. rhesus 43, inoculated May 21, 1926, under ether anesthesia, with the citrate blood withdrawn on the same day from *M. rhesus* 33. Intradermal inoculations were made on both eyebrows and on both sides of the abdomen. Blood was also applied to scarified areas on the eyebrows and abdomen, and 0.5 cc. was injected intravenously.

The animal showed no febrile reaction at any time during the 45 days of observation (Chart 5). Blood cultures made on June 26 were negative. Small nodules, 2 × 2 mm., appeared at each of the two sites of intradermal inoculation within about 24 days. They showed no change during the ensuing 10 days but

subsequently gained in size rather rapidly. They were situated subcutaneously, were perfectly round, and had a semitransparent, bluish, pearl-like color. On July 1 there were noticed about the periphery the minute red streaks which *Bartonella* lesions of this type show just before vascularization. One of the nodules excised at this time (July 1) yielded cultures in a dilution of 1:100,000. By Aug. 6, 1926, the lesions had all healed.

The microorganism obtained in culture from a monkey inoculated with human verruga tissue (Case P. 5) was capable, therefore, of inducing in *rhesus* monkeys a local and systemic infection the manifestations of which were in all respects similar to those obtained by inoculation of the strain of *Bartonella bacilliformis* from Oroya fever, *i.e.*, a prolonged course of irregular, remittent fever, during which the intracorpuscular parasites are present in the blood, and a skin lesion characterized by extensive proliferation of endothelial cells and by the presence of the parasites in the cytoplasm of these cells. The infection in the second passage animal (Monkey 41) was of the severe type; there was absolute anemia and hyperleucocytosis, and the local lesions were very large. The organisms were present in large numbers at one period of the disease, but only degenerated forms were found at a later stage. The blood from which the culture was obtained, however, induced only a mild, slowly developing infection, with no fever.

The local inoculation of nodular tissue from Monkey 41 into Monkey 42 (third passage) induced a severe local reaction and a febrile reaction of brief duration, during which the blood culture titer was as high as 1:1,000. As stated previously, direct microscopic detection of the parasites in the blood was unsuccessful in the case of mild infections. The cultural procedure was by far the most delicate means of diagnosis.

Cultural and Morphological Characters of the Microorganisms from Verruga.

The character of the lesions of *M. rhesus* 41 and the microscopic findings in the blood of this animal appear to leave no doubt that the organism injected was identical in pathogenic properties with the strain of *Bartonella bacilliformis* previously obtained from Oroya fever, a conclusion which is further substantiated by cultural and morphological study.

The first culture was obtained on the so called leptospira medium, which was inoculated with various dilutions of citrated blood of *M. rhesus* 33 and kept at 25°C. Growth became recognizable macroscopically within a week, the uppermost layer of the medium showing some grayish, minute particles; these gradually increased in number during subsequent weeks (Fig. 19). Under the dark-field microscope these particles were seen to consist of numerous tightly packed masses of irregular minute bacillary bodies. Individual organisms were occasionally seen, but the chief characteristic of the organisms is their constant tendency to form firm aggregates difficult to disperse (Fig. 21). Free flagella are seen in the agar mass.

On the surface of blood agar round raised colonies of variable size, the smallest almost microscopic (Fig. 20), the largest having the appearance of fine granules, are formed within about 6 to 8 days and after a few days reach 1 mm. or more in diameter, but seldom 2 mm. A light grayish color is noticeable when the light falls obliquely on the culture. The colonies are firm and are peeled off the agar readily by a platinum loop. It is difficult to break them up in fluid to form a uniform suspension. Under the dark-field microscope masses of agglomerated organisms are seen to predominate (Fig. 22); a few single or paired individuals may be found, and these are usually motile. Locomotion is in one direction only, but rotatory movement is also seen.

The organisms are Gram-negative, pleomorphic, and stain unevenly with Giemsa's solution. When stained a long time they appear reddish and ill defined in contour; they stain much less intensely than most bacteria. Special staining reveals two or more flagella attached to one end of the organism (Fig. 24), the length of the normal flagella varying from 2 to 5 μ. Abnormal detached flagella may reach a length of 20 to 30 μ.

Individuals in young cultures are more uniform in size and have a definite contour. Short rods, 0.3 to 0.4 μ wide and 0.45 to 1.5 μ in length, predominate. In older cultures the individuals are much more irregular in size and form, and numerous granular elements measuring less than 0.2 to 0.5 μ are found in masses; these stain intensely and have very indefinite outlines, the appearance simulating that of degenerative changes. Exceptionally long rods 2.5 to 3 μ may be found. There is no polar staining.

The optimum temperature for growth is 25–28°C.; at 37°C. growth ceases within 4 to 5 days. The optimum reaction of the medium lies between pH 7.8 and pH 8.

For the cultivation of this organism the addition of animal blood or serum and hemoglobin to solid or semisolid media is essential. No satisfactory fluid medium has so far been found. The organism is an obligate aerobe. The cotton plugs of culture tubes may be impregnated with paraffin, but sealing them hermetically with sealing wax prevents growth.

Like the strain of *Bartonella bacilliformis* from Oroya fever, the organism is non-spore-forming and hemophilic. It is provided with

TABLE I.

Anti-Oroya serum	Antigens	
	Oroya	Verruga
	0.5 cc.	0.5 cc.
cc.		
0.1	++++	++++
0.01	++++	++++
0.001	++	−
0.0001	−	−
0	−	−

++++ = complete fixation.
++ = 50 per cent hemolysis.
− = complete hemolysis.

several unipolar flagella of the characteristic bacterial type. A distinct cell wall, such as is seen in bacteria, can be recognized in young, actively motile forms.

The organism does not ferment any carbohydrate so far tested. Red corpuscles contained in the culture medium are not hemolyzed in the course of its growth. No putrefactive process seems to occur in the culture. Slight growth may be obtained on Loeffler's serum medium, but no liquefaction is observed.

Serological Identification of the Verruga Strain.

The morphological, cultural, and pathogenic properties of the verruga strain resemble so much those of the strain of *Bartonella*

bacilliformis obtained from a case of Oroya fever that the identity of the two organisms appeared extremely probable. Preliminary serological tests confirmed this conclusion.

A rabbit was repeatedly inoculated intravenously with saline suspensions of cultures of *Bartonella bacilliformis* during a period of 4 months, 1 to 2 cc. being injected at intervals of 4 to 5 days, and the serum obtained from the animal was tested for complement fixation with its homologous strain and also with the strain from verruga. Saline suspensions of colonies removed from the surface of blood slant cultures after 14 days growth at 25°C., and washed twice with saline solution, served as antigens. The suspensions were rather granular in appearance, owing to the difficulty of breaking up the colonies. To 0.5 cc. of the antigen (this quantity did not bind complement) were added the immune serum in quantities of 0.1, 0.01, 0.001, or 0.0001 cc., and 0.04 cc. guinea pig serum as complement. Two hemolytic units of anti-sheep amboceptor were used. Controls with normal rabbit serum were made in each instance. The total volume in each case was made up to 1.2 cc. with saline solution. The results are shown in Table I.

The results of the complement fixation tests show that there is an undeniably close serological relationship between the two strains, although the immune serum reacted more strongly with the Oroya than with the verruga strain.

SUMMARY AND CONCLUSIONS.

A saline suspension of a subcutaneous nodule excised from a verruga patient, and kept in the refrigerator for 14 days, on inoculation into two young *Macacus rhesus* monkeys (Nos. 33 and 34) induced irregular febrile reactions and enlargement of the lymph glands, and in one instance a subcutaneous nodule arose, independently of direct inoculation, on the tail. A microorganism has been isolated from the blood of both animals, and from the experimental nodule, which in pathogenic properties and in cultural and morphological characteristics is indistinguishable from the strain of *Bartonella bacilliformis* isolated from a case of Oroya fever.

The spontaneous skin lesion of Monkey 34 and the subcutaneous nodules induced by intradermal inoculation of cultures of the microorganism were histologically typical of experimental verruga lesions in monkeys and identical with the skin lesions induced in monkeys by *Bartonella bacilliformis*. The organism, like *Bartonella bacilliformis*, is an intracellular parasite, being found in the cytoplasm of the prolif-

erating endothelial cells of the lesions and in the erythrocytes of the blood.

The same variations in the manifestations of disease which have been noted in experimental infection with *Bartonella bacilliformis* were observed in the experimental verruga infection. In the second passage (Monkey 41) the infection induced by local inoculation of cultures was severe both locally and constitutionally and was accompanied by marked anemia. The organisms were found in the red cells in large numbers. In the third passage the systemic infection was less severe, but the local lesions were more striking.

Detection of the parasites in the blood is far more certain by the cultural method than by microscopic examination, the latter procedure being successful only in rather severe infections. The result of blood culture is therefore the decisive method in the final diagnosis of the disease.

Preliminary serological study shows that the organism isolated in the present instance from the skin lesion of a verruga patient and that previously obtained from the blood of a case of Oroya fever belong to the same serological group.

The data obtained justify the conclusion that verruga peruana is caused by *Bartonella bacilliformis*. They also definitely establish the fact that the inoculation of blood or sanguineous exudate from lesions of verruga peruana is capable of inducing in susceptible individuals a severe febrile systemic infection, such as that to which Carrion succumbed. The designation "Carrion's disease"[6] is therefore the appropriate one for both forms of the infection.

Bartonella bacilliformis may be regarded as a bacterium, since it has the essential features of that group of microorganisms.

EXPLANATION OF PLATES.

PLATE 2.

FIG. 1. *M. rhesus* 41, showing three verruga nodules on the right eyebrow 30 days after intradermal inoculation. The two nodules on the inner side had arisen at the site of removal of the initial nodule 16 days previously. Natural size.

FIG. 2. Verruga organisms in the red corpuscles of *M. rhesus* 41, 30 days after inoculation. × 1,500.

FIG. 3. Verruga organisms in the red corpuscles of the same monkey 52 days

after inoculation. They took a more reddish stain at this time and were less definite in outline, probably because they were in process of degeneration. Anemia was also most marked at this time. Subsequent attempts to find the parasite in the blood were unsuccessful. × 1,500.

FIG. 4. Verruga organisms in the nodule of *M. rhesus* 41, removed 14 days after inoculation. Giemsa's stain after fixation in Regaud's fluid. × 1,500.

FIG. 5. Verruga organisms in the nodule of a patient suffering with verruga peruana. Courtesy of Professor R. P. Strong. Giemsa's stain, after fixation in Zenker's fluid. × 1,500.

FIG. 6. Early lesions of experimental verruga in *M. rhesus* 42, 16 days after inoculation of a scarified area on the right eyebrow with a suspension of the nodule from *M. rhesus* 41. Natural size.

FIG. 7. Same lesions 23 days after inoculation.

FIG. 8. Early lesions on the scarified skin of the abdomen of the same monkey 16 days after inoculation. Natural size.

FIG. 9. Same lesions 23 days after inoculation.

FIG. 10. Subcutaneous nodule which arose spontaneously on the tail of *M. rhesus* 34, 59 days after the animal had been inoculated with a suspension of a nodule of the verruga patient P. 5. Natural size. For histological appearance of the nodule see Fig. 11.

PLATE 3.

FIG. 11. Histological appearance of the subcutaneous nodule on the tail of *M. rhesus* 34, 69 days after inoculation. (The gross appearance of the nodule is shown in Fig. 10.) Giemsa's stain after fixation in Regaud's fluid. × 152.

FIG. 12. Histological appearance of the nodule on the eyebrow of *M. rhesus* 41, removed 14 days after inoculation. Giemsa's stain after Regaud's fixation. × 152.

FIG. 13. Verruga organisms in the nodule of *M. rhesus* 41. Same section as shown in Fig. 12. × 1,000.

FIG. 14. Verruga organisms in the red blood corpuscles of *M. rhesus* 41. Eight different fields have been placed side by side. In some of them are seen red corpuscles containing one or more organisms. Blood films made 30 days after inoculation. Giemsa's stain. × 1,000.

FIG. 15. Blood of the same monkey 52 days after inoculation. The organisms appear somewhat degenerated but quite numerous. Giemsa's stain. × 1,000.

FIG. 16. *Bartonella bacilliformis* in the blood of an Oroya fever patient, for comparison (Patient S. A. 15, case from which *B. bacilliformis* was cultivated). Giemsa's stain. × 1,000.

PLATE 4.

FIG. 17. Verruga nodule from Case P. 5, fixed in Regaud's fluid and stained with Giemsa's solution, 14 days after removal from patient. The characteristic verrucous structure is still recognizable, although many cells have undergone degenera-

tion. The dark masses of irregular size are aggregates of minute bacilliform organisms. × 152.

FIG. 18. The same section as that shown in Fig. 17 but under a higher magnification. × 1,000.

FIG. 19. Appearance of colonies of the verruga organisms in the upper portion of a tube of leptospira medium. 28 days old at 25°C. Natural size.

FIG. 20. Appearance of colonies of the verruga organisms on the surface of horse blood agar slant, 12 days old at 25°. Natural size.

FIG. 21. Dark-field view of the culture shown in Fig. 19. × 1,000.

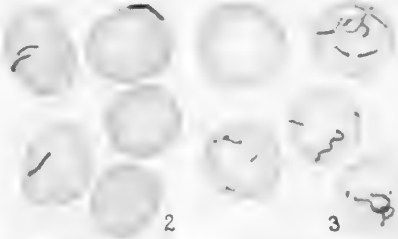
FIG. 22. Dark-field view of the culture shown in Fig. 20. × 1,000.

FIG. 23. The verruga organisms from a blood agar slant culture, 11 days old at 25°C. Giemsa's stain. × 1,000.

FIG. 24. Flagella of the verruga organisms. Zettnow's stain. × 2,000.

Intradermal inoculation, 30 days.

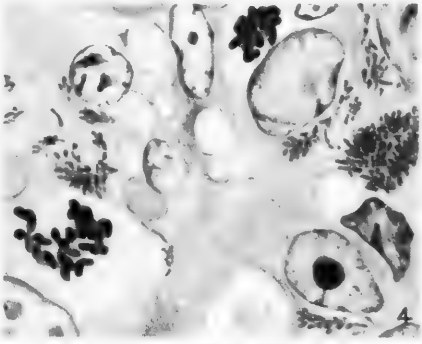
Scarification, 16 days.



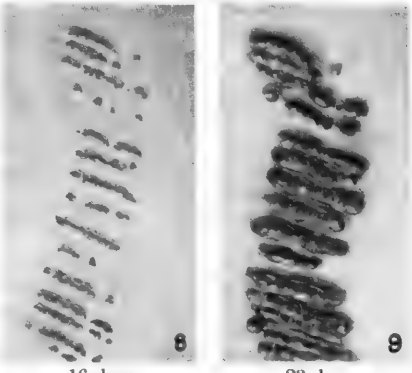
Blood, 30 days.

Blood, 52 days.

Scarification, 23 days.

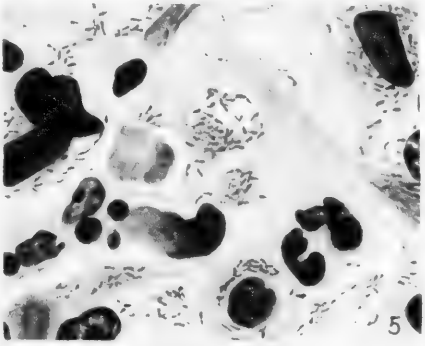


Eyebrow nodule, 14 days.



16 days.

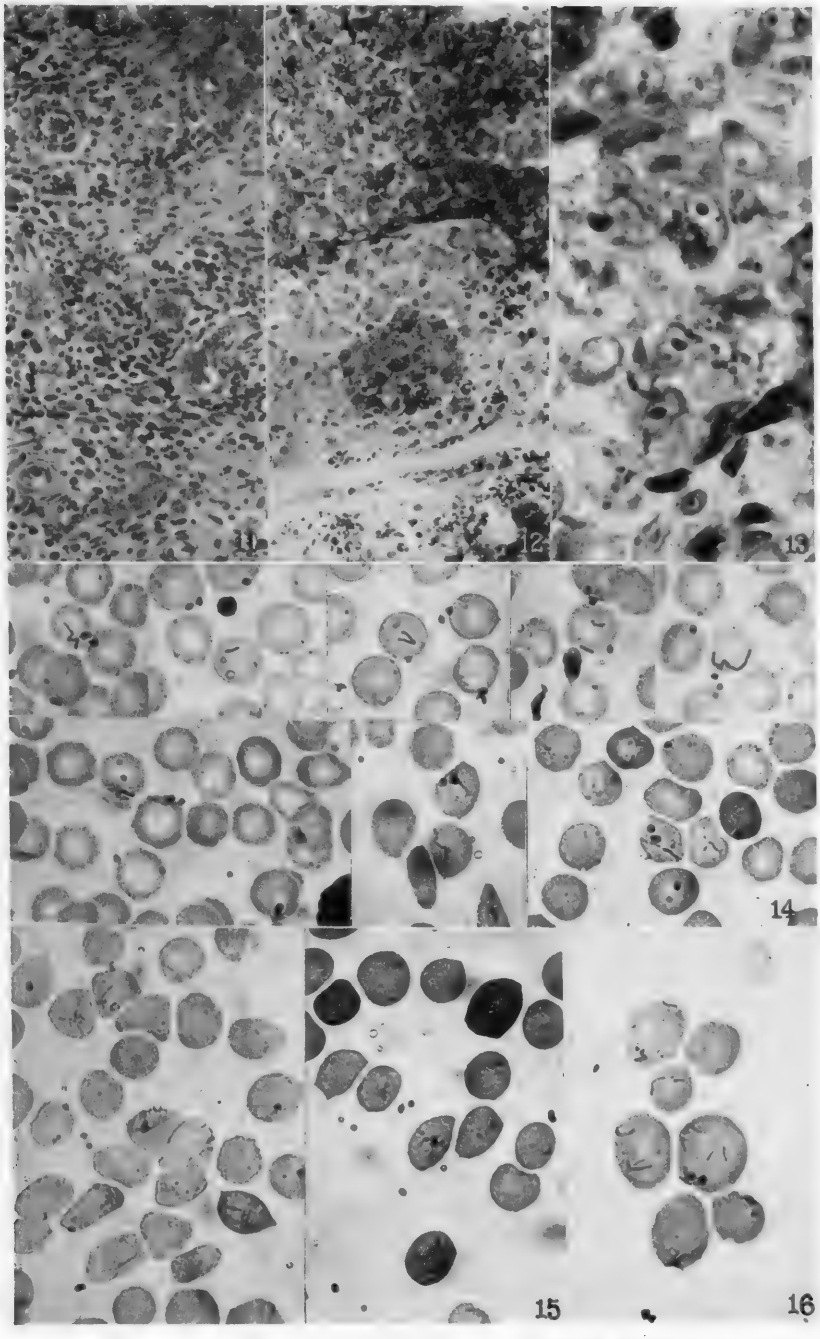
23 days.



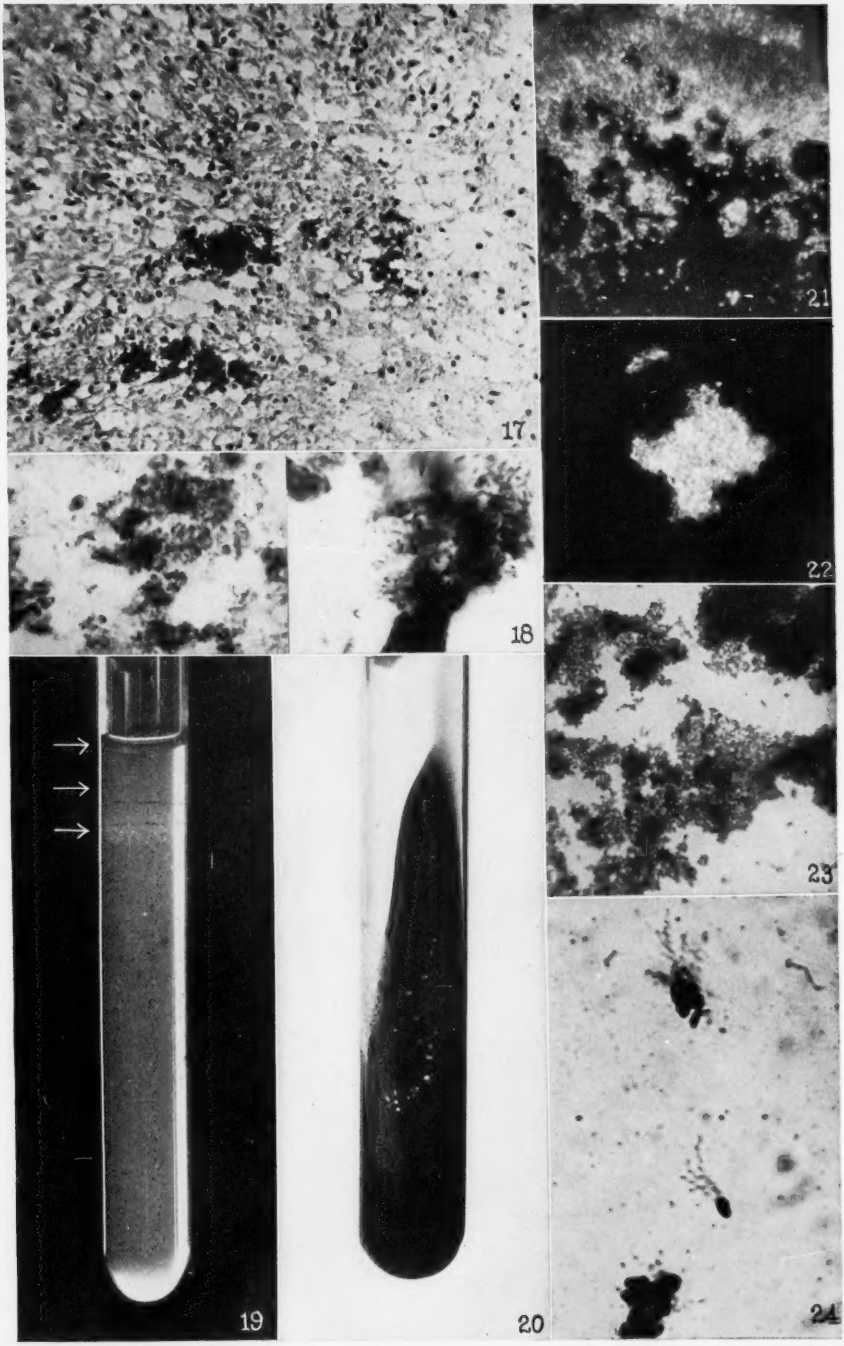
Human nodule.

Nodule on tail.

(Noguchi: Etiology of verruga peruana.)



(Noguchi: Etiology of verruga peruana.)



(Noguchi: Etiology of verruga peruana.)